"BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF EZETIMIBE IN HUMAN PLASMA BY LC/MS/MS"

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Submitted by

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CERTIFICATES

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF EZETIMIBE IN HUMAN PLASMA BY LC/MS/MS"** submitted by the student bearing **REG.No.261630204** to "**The Tamil Nadu Dr. M.G.R. Medical University – Chennai**", in partial fulfilment for the award of Degree of **Master of Pharmacy** in **Pharmaceutical Analysis** was evaluated by us during the examination held on.....

Internal Examiner **External Examiner**

CERTIFICATE

This is to certify that the work embodied in this dissertation Entitled **"BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF EZETIMIBE IN HUMAN PLASMA BY LC/MS/MS"** submitted to "**The Tamil Nadu Dr. M.G.R. Medical University-Chennai**", in partial fulfilment and requirement of university rules and regulation for the award of Degree of **Master of Pharmacy** in **Pharmaceutical Analysis**, is a bonafide work carried out by the student bearing **REG.No.261630204** during the academic year 2017-2018, under the guidance and supervision of **Mr. D. KAMALA KANNAN, M.Pharm.,** Assistant Professor, Department of Pharmaceutical Analysis, J.K.K. Nattraja College of Pharmacy, Komarapalayam.

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I do hereby declared that the dissertation **"BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF EZETIMIBE IN HUMAN PLASMA BY LC/MS/MS"** submitted to "**The Tamil Nadu Dr. M.G.R Medical University - Chennai",** for the partial fulfilment of the degree of **Master of Pharmacy** in **Pharmaceutical Analysis,** is a bonafide research work has been carried out by me during the academic year 2017-2018, under the guidance and supervision of **Mr. D. KAMALA KANNAN, M.Pharm.,** Assistant Professor, Department of Pharmaceutical Analysis, J.K.K. Nattraja College of Pharmacy, Komarapalayam.

I further declare that this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associateship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

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Dedicated to

Parents,

Teachers&

My Family

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LIST OF CHROMATOGRAMS

LIST OF ABBREVIATION USED

µg	Microgram
µL	Micro liter
a	Slope
amu	Atomic mass unit
AQ	Aqueous
b	Intercept
CAD	Collisional Activated Dissociation gas
CC	Calibration curve
CE	Collision energy
Conc.	Concentration
CUR	Curtain gas
CV	Coefficient of variation
CXP	Cell exit potential
DF	Dilution factor
DP	Declustering potential
EDTA	Ethylene diamine tetraacetic acid
EP	Entrancing potential
FP	Focusing potential
GS1	Gas source 1
GS2	Gas source 2
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
HQC	High quality control
hr	Hour
IS	Internal standard
ISP	Ion source parameter
LC-MS/MS	Liquid chromatography coupled with
LOQ	Limit of quantification

LOQ QC	Limit of quantification quality control
LQC	Low quality control
min	Minute
mL	Milliliter
MQC	Medium quality control
MRM	Multiple reaction monitoring
MV	Method validation
NAP	Not applicable
ng	Nanogram
M1QC	Medium 1 quality control
No.	Number
p	Pressure
p.a.r.	Peak area ratio
QC	Quality control
Q1	Quadrupole 1
Q3	Quadrupole 3
RT	Room temperature
r^2	Correlation coefficient
rpm	Revolutions per minute
SD	Standard deviation
sec	Seconds
SOP	Standard operating procedure
SS	System suitability
STD	Standard
TEM	Temperature
ULOQ	Upper limit of quantitation
V	Volume
x	Representation of concentration
y	Representation of peak area ratio

INTRODUCTION

1.0 BIOANALYTICAL AND BIOEQIVALANCE STUDIES

The design, performance, and evaluation of bioavailability and bioequivalence studies have received major attention from academia, the pharmaceutical industry and health authorities over the last couple of decades. Comparison of therapeutic performance of two medicinal products containing the same active substance is critical for assessing the possibility of supplanting an innovator with any essentially similar medicinal product. In practice, demonstration of bioequivalence is generally the most appropriate method of substantiating therapeutic equivalence between medicinal products. (Rechard F. Venn 2004).

This thesis deals with the **"**Bioanalytical Method Development and Validation for the Estimation of Ezetimibe in Human Plasma by LC-MS/MS**".** Before discussing the experimental results, a brief introduction to Biopharmaceutical analysis, analysis of drugs and metabolites in biological media, Preliminary treatment of biological samples, Extraction procedures for drugs and metabolites from biologic samples, estimation of drugs in biological sample by LC-MS, quantitative techniques in LC-MS, Bioavailability(BA) and Bioequivalence studies(BE), Methods to document BA and BE, Comparison of BA measures in BE studies, Moieties to be measured in BA and BE studies, and literature survey will be briefly reviewed here. Biopharmaceutical analysis deals with determining trace (microgram or less) levels of organic and selected inorganic medicaments in biologic fluids.

The drugs are absorbed orally and peak plasma concentration is reached at a certain time. The drug is subsequently distributed, metabolized, and excreted and the drug concentration declines over a period of time depending on the elimination half-life. The bioequivalence studies conducted to investigate the pharmacokinetic parameters of two pharmaceutical formulation of the same drug and to demonstrate the equivalence of their pharmacokinetic parameters.

Bioavailability Studies (BA) focus on determining the process by which a drug is released from the oral dosage form and moves to the site of action. BA data provide an estimate of the fraction of the drug absorbed, as well as its subsequent distribution and elimination. BA can be generally documented by a systemic exposure profile obtained by measuring drug and/or metabolite concentration in the systemic circulation over time.

Bioequivalence studies (BE) involves comparison between a test (T) and reference (R) drug product, where T and R can vary, depending on the comparison to be performed (e.g. to be marketed dosage form versus clinical trial material, generic drug versus reference listed drug, drug product changed after approval versus drug product before the change). For two orally administered drug products to be bioequivalent, the active drug ingredient or active moiety in the test product should exhibit the same rate and extent of absorption as the reference drug product. BE information is required to ensure therapeutic equivalence between a pharmaceutically equivalent test drug product and a reference listed drug.

1.1 NEED FOR BIO-EQUIVALENCE STUDIES

1.1.1 Applications for products containing new active substances

During the development of a new active substance (new chemical entity) intended for systemic action, bioequivalence studies are necessary as bridging studies between,

(i) Pivotal and early clinical trial formulations;

(ii) Pivotal clinical trial formulations,

Especially those used in the dose finding studies, and the to-be-marketed medicinal product. (Dieter Hauschke and Volker, 2006)

1.1.2 Applications for products containing approved active substances

In-vivo bioequivalence studies are needed when there is a risk that possible differences in bioavailability may result in therapeutic in-equivalence. The Committee for Proprietary Medicinal Products (CPMP) guidance (2001) devotes an entire section to the necessity of bioequivalence studies for various dosage forms, taking into consideration the concepts underlying the Biopharmaceutics Classification System, i.e., high solubility, high permeability for the active substance, and high dissolution rate for the medicinal product.

This section also addresses special topics such as

- Exemptions from bioequivalence studies in the case of oral immediate release forms (in vitro dissolution data as part of a bioequivalence waiver) Post approval changes.

- Dose proportionality of immediate release oral dosage forms (bioequivalence assessment for only one dose strength).

- Suprabioavailability (which necessitates reformulation to a lower dosage strength, otherwise the suprabioavailable product may be considered as new medicinal product, the efficacy and safety of which have to be supported by clinical studies).

1.1.3 Applications for modified release forms essentially similar to a marketed modified release forms

The requirements for modified release forms are stated in the CPMP Note for Guidance on Modified Release Oral and Transdermal Dosage Forms (1999), which differentiates between prolonged, delayed and transdermal release forms. Prolonged release formulations can be assessed as bioequivalent on the basis of single-dose and multiple-dose studies, which are designed to demonstrate that

- The test formulation exhibits the claimed prolonged release characteristics of the reference.

- The active drug substance is not released unexpectedly from the test formulation (dose dumping).

- Performance of the test and reference formulation is equivalent after single dose and at steady state.

- The effect of food on the in vivo performance is comparable for both formulations when a single-dose study is conducted comparing equal doses of the test formulation with those of the reference formulation administered

immediately after a predefined high fat meal. This study should be conducted with the same strength(s) as those of the pivotal bioequivalence studies.

In the case of prolonged release single unit formulations with multiple strengths, a single-dose study under fasting conditions is required for each strength. Studies at steady state may be conducted with the highest strength only, if certain criteria for extrapolating bioequivalence studies (linear pharmacokinetics, same qualitative composition, etc.) are fulfilled.

For multiple unit formulations of a medicinal product showing linear pharmacokinetics with multiple strengths, a single-dose study under fasting conditions on the highest strength is sufficient, provided that the compositions of the lower strengths are proportional to that of the highest strength, the formulations contain identical beads or pellets, and the dissolution profiles are acceptable.

For delayed release formulations, postprandial bioequivalence studies are necessary as food can influence the absorption of an active substance administered in an enteric-coated formulation.

The bioequivalence of a transdermal drug delivery system (TDDS) in comparison to the innovator's product should usually be assessed after single dose as well as after multiple dose administration. When marketing authorization of multiple strengths is required, the bioequivalence study can be performed with the highest dosage strength provided that exact proportionality in the formulation is given, i.e., the composition is the same, and the strength is proportional to the effective surface area of the patch, and that there is an acceptable in vitro release test (CPMP, 1999).

2.0 FACTORS TO BE CONSIDERED

2.1 Storage Requirements for Biologic Samples

In order to avoid decomposition or other potential chemical changes in the drugs to be analyzed, biologic samples should be frozen immediately upon collection and thawed before analysis. When drugs are susceptible to plasma esterase, the addition of esterase inhibitors, such as sodium fluoride, to blood samples immediately after collection helps to prevent drug decomposition.

When collecting and storing biologic samples, the analyst should be varying of artefacts from tubing or storage vessels that can contaminate the sample. For example, plastic-ware frequently contains the high boiling liquid Bis-(2-ethylhexyl) phthalate, similarly, the plunger-plugs of vacutainers are known to contain tri-butoxyethyl phosphate, which can interfere in certain drug analyses.

In the case of faeces, lyophilization of the sample before storage is highly desirable unless prior investigations have revealed little or no reactivity of the drug components with the endogenous intestinal micro-organisms.

2.2 Preliminary Treatment of Biologic Samples

In most cases, preliminary treatment of a sample is needed before the analyst can proceed to the measurement step. Analyses are required for drug in samples as diverse as plasma, urine, faeces, saliva, bile, sweat, and seminal fluid. Each of these samples has its own set of factors that must be considered before an appropriate pretreatment method can be selected. Such factors as texture and chemical composition of the sample, degree of drug-protein binding, chemical stability of the drug, and types of interferences can affect the final measurement step.

2.2.1 Protein Precipitation or Denaturation

Biologic materials such as plasma, feces, and saliva contain significant quantities of protein, which can bind a drug. The drug may have to be free from protein before further manipulation. Protein Denaturation is important, because the presence of proteins, lipids, salts, and other endogenous material in the sample can cause rapid deterioration of HPLC columns and also interfere the assay. Protein denaturation procedures include the use of tungstic acid, ammonium sulfate, heat, alcohol, trichloroacetic acid, and perchloric acid.

Methanol and acetonitrile frequently have been used as protein denaturants of biologic samples. Methanol sometimes is preferred because it produces a flocculent precipitate and not the gummy mass obtained with acetonitrile. Methanol also gives a clearer supernate and may prevent the drug entrapment that can be observed after acetonitrile precipitation.

Ultrafiltration and dialysis procedures also have been used to remove proteins from biologic fluids. These procedures are not widely used because they are slow.

2.2.2 Hydrolysis of Conjugates

The presence-of drug metabolites as conjugates, such as glucuronides and sulfates, in biologic samples cannot be ignored. The effect of a drug depends to a considerable extent on the biotransformation that occurs in the human body. Therefore, it may be important to isolate the actual conjugates. Samples containing either glucuronide acetals or sulfate esters are usually pretreated using enzymatic or acid hydrolysis. The unconjugated metabolites that result from the hydrolysis

procedure are less hydrophilic than their conjugates and usually can be extracted from the biologic matrix.

A nonspecific acid hydrolysis can be accomplished by heating a biologic sample for 30 min at 90 to 100°C in 2 to 5N hydrochloric acid. Upon cooling, the pH of the sample can be adjusted to the desired level and the metabolite removed by solvent extraction. Particularly stable conjugates sometimes require hydrolysis in an autoclave.

2.2.3 Homogenization

For samples containing insoluble protein, such as muscle or other related tissues, a homogenization or solubilizing step using 1N hydrochloric acid may be required before treating the sample further. For gelatinous samples such as seminal fluid or sputum, liquefaction is achieved via sonication. A solid sample such as feces can be homogenized with a minimum amount of methanol. Homogenization is usually performed with a blade homogenizer (e.g., Waring Blender).

3.0 EXTRACTION PROCEDURES FOR DRUGS AND METABOLITES FROM BIOLOGIC SAMPLES

After pre treating biologic material, the next step is usually the extraction of the drugs from the biologic matrix. All separation procedures use one or more treatments of matrix-containing solute with some fluid. If the components are a liquid (extracting solvents) and a solid (e.g., lyophilized feces), it is an example of liquid-solid extraction. If the extraction involves two liquid phases, it is an example of liquid-liquid extraction. (Richard F.Venn 2004)

3.1 Liquid-Solid Extraction

Liquid - solid extractions occur between a solid phase and a liquid phase, either phase may initially contain the drug substance. Among the solids that have been used successfully in the extraction (usually via adsorption) of drugs from liquid samples are XAD-2 resin, charcoal, alumina, silica gel, and aluminum silicate.

Sometimes the drugs are contained in a solid phase, such as in lyophilized specimens. Liquid-solid extraction is often particularly suitable for polar compounds that would otherwise tend to remain in the aqueous phase. The method could also be useful for amphoteric compounds that cannot be extracted easily from water.

Factors governing the adsorption and elution of drugs from the resin column include solvent polarity; flow rate of the solvent through the column, and the degree of contact the solvent has with the resin beads. In the adsorption process, the hydrophobic portion of the solute that has little affinity for the water phase is preferentially adsorbed on the resin surface while the hydrophilic portion of the solute remains in the aqueous phase. Alteration in the lipophilic / hydrophilic balance within the solute or solvent mix, and not within the resin, affects adsorption of the solute. Biologic samples can be prepared for cleanup by passing the sample through the resin bed where drug (metabolite) components are adsorbed and finally eluted with an appropriate solvent. The liquid solid extraction method provides a convenient isolation procedure for blood samples, thus avoiding solvent extraction, protein precipitation, drug losses, and emulsion formulation. It is possible, however, that strong drug-protein binding could prevent sufficient adsorption of the drug to resin.

3.2 Dehydration Methods

An aqueous biologic sample is treated with a sufficient quantity of anhydrous salt (sodium or magnesium sulfate) to create a "dried" mix. This mix is then extracted with a suitable organic solvent to remove the desired drug or metabolite.

3.3 Liquid-Liquid Extraction

Liquid-liquid extraction is probably the most widely used technique because

- The analyst can remove a drug or metabolite from larger concentrations of endogenous materials that might interfere with the final analytic determination.
- The technique is simple, rapid, and has a relatively small cost factor per sample.
- The extract containing the drug can be evaporated to dryness, and the residue can be redissolved in a smaller volume of a more appropriate solvent. In this manner, the sample becomes more compatible with a particular analytic methodology in the measurement step, such as a mobile phase in HPLC determinations.
- The extracted material can be redissolved in small volumes (e.g., 100 to 500 µl of solvent), thereby extending the sensitivity limits of an assay.
- It is possible to extract more than one sample concurrently.
- Near quantitative recoveries (90% or better) of most drugs can be obtained through multiple or continuous extractions.

Partitioning or distribution of a drug between two possible liquid phases can be expressed in terms of a partition or distribution coefficient, usually called P. A partition coefficient is constant only for a particular solute, temperature, and pair of solvents used. By knowing the P value for the extracted drug and the absolute volumes of the two phases to be utilized, the quantity of drug extracted after a single extraction can be obtained. In multiple extractions methodology, the original biologic sample is extracted several times with fresh volumes of organic solvent until as much drug as possible is obtained. Because the combined extracts now contain the total extracted drug, it is desirable to calculate the number of extractions necessary to achieve maximum extraction.

3.3.1 Factors Affecting the Partition Coefficient

Factors that influence partition coefficient and hence recovery of drugs in liquid-liquid extraction are choice of solvent, pH, and ionic strength of the aqueous phase. In almost all cases, one of the liquid phases is aqueous because of the nature of a biologic sample. The second liquid is selected by the analyst. It is highly desirable to select an organic solvent that shows great affinity for the drug analyzed, yet leaves contaminants or impurities in the aqueous or biologic phase. The solvent should be immiscible with an aqueous phase, should have less polarity than water, and should solubilize the desired extractable compound to a large extent. It should also have a relatively low boiling point so that it can be easily evaporated if necessary. Other considerations are cost, toxicity, flammability, and the nature of the solvent. If larger numbers of samples are to be extracted, the volume of solvent needed per sample can affect the overall cost of the assay procedure.

It is generally accepted that diethyl ether and chloroform are the solvents of choice for acidic and basic drugs, respectively, especially when the identity of the drugs in the samples is unknown. In these cases, any chemically neutral drugs are extracted into either solvent depending on their relative partition tendencies. Proper pH adjustment of a biologic sample permits quantitative conversion of an ionized drug to an un-ionized species, which is more soluble in a nonpolar solvent and therefore, extractable from an aqueous environment. In analyses that must determine a known drug or metabolite, the proper pH for extraction can be calculated from the Henderson-Hasselbalch equation using the pKa of the compound. If the species to be analyzed is unknown, the pH must be approximated based on the chemical nature of the suspected agent.

Third Factor influencing extractability of drugs from biologic samples is ionic strength. Addition of highly water-soluble ionized salts, such as sodium chloride, to an aqueous phase creates a high degree of interaction between the water molecules and the inorganic ions in solution. Fewer water molecules are free to interact with a unionized drug.

Therefore, the solubility of the drug in the aqueous phase decreases, thereby increasing the partitioning or distributing in favor of the non-polar or organic phase. The technique is commonly called “salting out”. Either mechanical or manual tumbling, rocking, or vigorous shaking of the samples can accomplish mixing of the aqueous-organic phases. The percent recovery of a drug vs. time and/or type of mixing should be investigated for each biologic sample. In many cases, vigorous shaking of a sample should be avoided because it leads to emulsification, which can be intractable to centrifugation. Emulsification is often observed when organic

solvents are used at basic pH whereas certain organic solvents such as n-hexane and diethyl ether are less emulsion-prone.

Certain types of amphoteric drugs or drugs that possess extreme water solubility are not amenable to classic solvent extraction. In these cases, other types of analytic methodology such as ion-pairing must be adopted. The technique of back-extraction can be applied with success to the analysis of drugs in biologic samples. The purpose of the methodology is to further purify an extract by removing either drug or impurities by additional extractions.

4.0 ANALYTICAL TECHNIQUES IN BIOANALYSIS

Chromatography is a separation technique by which solutes of two or more components are separated by a dynamic differential migrational process. In a system consisting of two phases, one of which moves continuously in a given direction and in which individual components exhibit differential mobility due to difference in their adsorption or partition or molecular size etc.

Methods for separation of Drugs and their metabolites in biological sample can be developed, provided one has knowledge about the nature of the Drug, its molecular weight, polarity, pKa, ionic character and the solubility parameter. An exact recipe for HPLC, however, cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column to be used with what kind of mobile phase. Interfacing mass spectrometers with chromatographic separation techniques is one of the most sophisticated and sensitive technique used in detection and quantization of Drug and its active metabolite in biological fluid. (Dougles A Skoog, 1996)

4.1 Selection of Mobile Phase

Since mobile phase governs solute –stationary phase interaction

- Practical considerations dictate that it should not degrade the equipment or column packing .So strong acids; bases and halides should be avoided.
- Chemical purity of sample is important factor. Since large volume of solvents are pumped through the column. Trace amount of impurities can easily concentrate in column and eventually detrimental to the result. So HPLC grade solvents only recommended to use in the analysis
- Volatility should be considered if sample recovery is required and mass transfer between solvent and stationary phase will be reduced.

Water, acetonitrile, ethanol, 2-propanol & methanol are widely used solvents. High concentration of THF (Tetrahydrofuran) as modifier should be used with extreme caution in APCI (Atmospheric Pressure Chemical Ionization) with heated nebulizer due to flammability air should not be used or auxiliary gas in APCI operation.

4.2 Role of solvent type

Chromatographic separations thus vary with solvent properties and are related to sample solubility, polarity and solvent strength. Solvents that interact strongly with the sample will increase the sample ion exit in the solvents and are not able to equilibrate with adsorbent surface. Changing the organic solvent will change the selectivity. In reverse phase, less polar solvent exhibit greater solvent strength than polar solvents. The solvents water (most polar), methanol, Acetonitrile and Tetrahydrofuran placed in ascending order of their polarity.

4.3 Selection of buffer and role of pH

pH is another factor in resolution equation that will affect the selectivity of the separation. In reverse phase HPLC, sample retention increases when the analyte is more hydrophobic .Thus when an acid (HA) or base (B) is ionized (converted in the form of unionized free or base) it becomes more hydrophilic and is less interactive with column binding sites.

Thus when selecting a buffer for a given application the following considerations are important. The buffer capacity is dependent on the buffer pH, pKa and buffer concentration. Other properties such as volatility, solubility, stability of the buffer and its reactivity to the analytes play important role in chromatographic system.

- Ammonium formate & ammonium acetate are suitable for buffer mobile phase in LC/MS/MS. Usually 2-10 mM concentration is adequate but concentration up to 50 mM can be used.
- Ammonium acetate may be used to replace phosphate buffers, which are not recommended for LC/MS.
- Ammonium adducts will frequently be observed in positive ion operation and formate or acetate ion adduct will be observed in negative ion operation.
- All the ammonium buffers may enhance sensitivity of weakly acidic compound undergoing negative ion analysis.
- Basic compounds will usually show an enhanced signal by lowering pH of mobile phase. Strong, volatile acid such as formic acid, acetic acid,

Triflouroacetic acid are recommended in concentration 0.1 to 1.0 % usually to enhance MS ionization but LC requirement may need higher concentration.

- Addition of acid will suppress the negative ion signal intensity since weakly acidic compound will not be deprotonated in acidic solution.

Triflouroacetic acid may be used in LC/MS especially for protein / peptide analysis but positive ion signals intensity is usually lower when formic acid is used. TFA and sulfonic acids should not be used for negative ion operation. Triethylamine may be used but it may suppress the ionization of less basic compound in positive ion operation (it gives an enhanced intense ion at m/z 102). It may be used in negative ion mode to enhance ionization of other compounds.

4.4 Selection of column

HPLC column is the important tool for separation of analytes .So the column must possess good selectivity, efficiency and reproducibility to provide good separation of analytes. Commonly used column are C-18, C-8, Phenyl, Cyano columns. They are chemically different bonded phase having different selectivity with same mobile phase.

5.0 METHOD DEVELOPMENT

Method development involves considerable trial and error procedures.

- In general, one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water-soluble.

- The pH of the mobile phase has to be selected in such a way that the compounds are not ionized. If the retention times are too short, then decrease 5 % of the organic phase concentration in the mobile phase, if the retention times are too long, an increase 5% of organic phase concentration in the mobile phase is needed.

- Elution of Drug molecules can be altered by changing the polarity of the mobile phase. The elution strength of a mobile phase depends upon its polarity. Ionic samples (acidic or basic) can be separated only, if they are present in undissociated form. Dissociation of ionic samples may be suppressed by proper selection of pH.

- Whenever acidic or basic samples are to be separated it is strongly advisable to control mobile phase pH by adding a buffer. PH of the buffer is to be adjusted before adding organic phase. The buffer selected for a particular separation should be used to control pH over the range of pka ±1.0.

- Optimizations can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that all the compounds are detected by more or less symmetrical peaks on the chromatogram.

- During method development selection of column can be streamlined by starting with shorter columns (50 mm, 100 mm or even 150 mm). By selecting a shorter column with an appropriate phase the run time can be minimized so that an elution order and an optimum mobile phase can be quickly determined. The internal diameter of the column is also one factor is to be considered.

- Many laboratories use 4.6mm ID as standard one, but it is worth considering using 4mm ID column as an alternative. This 4 mm column requires only 75 % of the solvent consumption than that of 4.6 mm column.

- Selecting an appropriate stationary phase can also help to improve the efficiency of method development. For example, a C-8 phase (reversed phase) can provide a further time saving over a C-18 as it doesn't retain the analytes as strongly as the C-18 phase.

- For normal phase application cyano phases are most versatile. C-18 (250 x 4.6 mm) column are more often used in laboratory. These columns are able to resolve a wide variety of compounds due to their selectivity and higher number of theoretical plates.

- In bio-analysis the method development step additionally require the extraction trial in order to recover the analyte and internal standard from highly complicated biological matrix. One should have knowledge about the nature of the Drug, namely its molecular weight, polarity, pKa, ionic character and the solubility parameter.

- Selection of internal standard should be on the basis of structural similarity, physicochemical properties related to the analyte to be quantified.

Method optimization or Performance of selected method is checked by running three or more precision and accuracy batches and evaluating the results for meeting acceptance criteria for within run precision, accuracy and between run precision and accuracy.

Finally selected method has to be validated to see whether it does what it was intended to do; i.e. it must be validated through validation parameters. The validated bio-analytical method is then applied for quantitation of Drug and its metabolites at its LOQ level with suitable precision and accuracy in a reproducible manner.

6.0 INSTRUMENTATION

6.1 Liquid chromatography and mass spectrometry

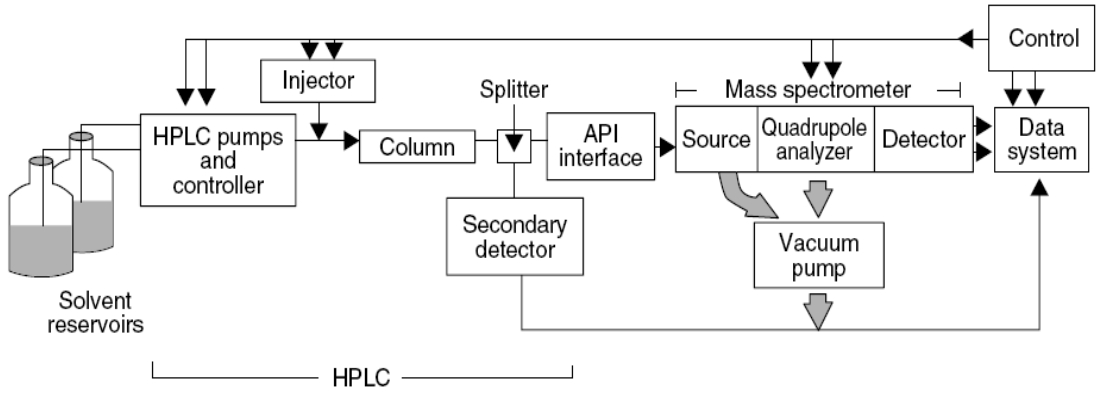
It is the way of interfacing the high vacuum domain of mass spectrometer with the condensed phase domain of liquid chromatography. (Marvin C. Mcmaster 2006)

6.2 Need of LC system interfacing with mass spectrometer

The separation is done prior to mass analysis because the mass spectrometer is incapable of directly determining every analyte in all type of sample. The Liquid chromatography can be regarded as a part of preparative procedure required for sample clean up which improves linearity, accuracy and better sensitivity. Mass spectrometer provides greater selectivity and sensitivity for chromatographic development because endogenous matrix can co-elutes with analytes yet not interferes as long as these components possess precursor masses.

The main purpose of interface is to evaporate the mobile phase and transfer the analytes from the higher pressure/atmospheric pressure at which chromatographic separation is achieved to the lower pressure required for the mass analysis. LC/MS is highly effective interface for coupling liquid chromatograph to the mass spectrometer. Mass spectrometer used as a detector system while liquid chromatograph taken as separation system.

BLOCK DIAGRAM OF LC – MS/MS



6.2.1 Sample inlet system

There are two opinions of a sample inlet system

- The sample introduced as neutral species through a controlled vacuum leak followed by ionization in vacuum chamber.
- Create the ion at atmospheric pressure and then introduced the ion in to the mass spectrometer through a controlled vacuum leak with aid of electrostatic this process is called API (atmospheric pressure ionization) provide best way when a dynamic coupling of liquid chromatograph done.

API is largely responsible for dramatic growth of mass spectrometry in pharmaceuticals industry, so API-LC/MS system is now-a-days commonly used.

6.2.2 Ionization source

Ionization proceeds by two fundamental processes:

- Loss/gain of an electron
- Loss/gain of a charge particle

An odd electron ions is generated by the gain/loss of an electron. In vacuum generating method ionic species of identical nominal molecular weight differ only by the mass of an electron to the neutral specie from which it was generated. An even electron is produced by gain or loss of even electron specie from a molecule.

There are four common modes of ionization

- Electron ionization (EI)
- Chemical ionization (CI)
- Matrix-Assisted Laser desorption Ionization (MALDI)
- Atmospheric pressure ionization (API)

In LC/MS interfacing Atmospheric Pressure Ionization (API) is the potential Ionization techniques because

- It gives softer ionization
- It provide convenient interface with liquid chromatograph

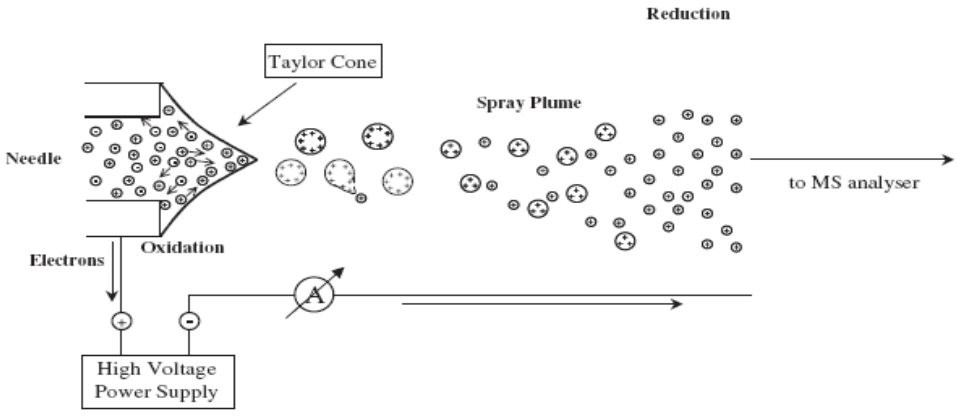
The less fragmentation of the molecular ions and a convenient interface with liquid chromatograph at ambient pressure to mass spectrometry at high vacuum.

6.2.2.1 Type of API Source

- Atmospheric Pressure Electro Spray Ionization
- Atmospheric Pressure Chemical Ionization

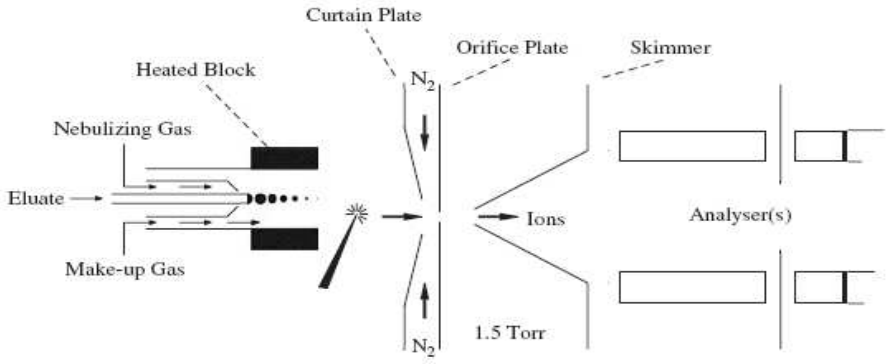
6.2.2.1.1 Atmospheric Pressure Electro Spray Ionization

It is an atmospheric ionization technique in which ions are generated in the solution phase by evaporation of carrier solvent and ions are produced in gas phase. An appropriate solvent from LC (liquid chromatographic) system is passed through a metal capillary to which a static DC voltage is applied to create ionization of effluents. When the solvent got evaporate the charge density increases creating columbic repulsion and subsequent dissociation of droplet. Further evaporation of droplet creates an environment in which charge transfer takes place from the solvent to the analytes. (Achille Cappiello 2006) Typically a voltage of 2.5 to 5 kV will be applied to generate an even electron ion in gas phase. This method is commonly used for high molecular compounds. Presently most of the designs rely on coaxial gas flow (nitrogen) which improves desolation. This source with coaxial gas flow are called nebulization assisted electro spray ionization. Low ionization is observed in this technique due to solvent clustering and analyte adduct formation, so this is most applicable for LC/MS system.



6.2.2.1.2 Atmospheric Pressure Chemical Ionization

It is an ionization technique in which the ionization occurs not in vacuum but at atmospheric pressure. It is a gas phase ionization process where by gas phase molecules are isolated from the carrier solvent before ionization. Generally less polar compounds are ionized by this method. **(Michaela Malm, 2000).**



Atmospheric Pressure Chemical Ionization

6.2.3 Mass analyzer

Any mass analyzer contains electrical or magnetic field or combination of two, which is capable of manipulating the trajectory of ions in a vacuum chamber. For any mass analyzer the analyte particle, should be charged in order to separate the ion from the other ions. There are so many type of analyzer used in MS system but specially Quadrupole analyzer is used frequently in the case of LC/MS/MS.

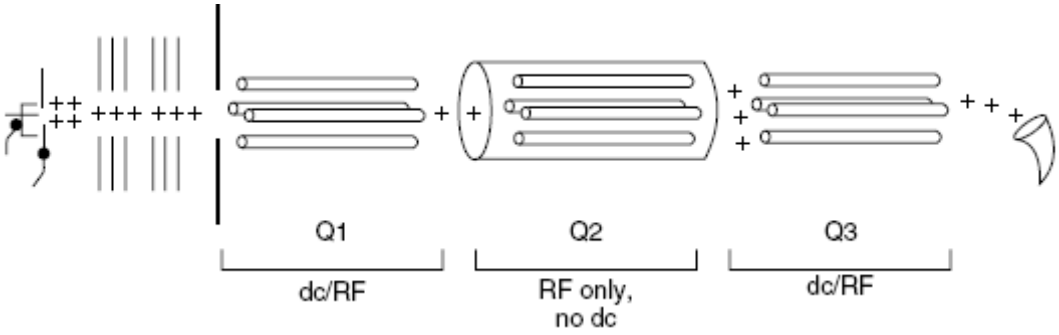
6.2.3.1 Quadrupole Mass Analyzer

It consists of two pair of electrically conducting rods/electrodes on to which a concurrent radio frequency (RF) and direct current (DC) voltage is applied. The rod consists of molybdenum or gold, diameter of 1 cm & 30 cm in length. Opposing quadrupole rods pairs are connected electrically such that they carry identical

RF/DC ratio. For a given RF/DC voltage ratio the quadrupole analyzer filter only the ion with in a selected mass/charge ratio have a stable trajectory as they pass from one quadrupole rod set to the other. If RF/DC is held constant only a narrow m/z range will transmit the quadrupole. In this configuration quadrupole mass analyzer act as a mass filter or quadrupole filter. In case of bioanalysis it is used as a tandem (MS/MS).

It consists of a capillary interface for liquid introduction, a heated nebulization system and a high voltage corona needle, it require high liquid flow rate like 200 micro liter/min.

Quadrupole Mass Analyzer



Nebulisation of effluent is done by heated gas flow (nitrogen) generating gas phase molecules of both (effluent and analyte). A metal needle is placed between nebulization probe and first vacuum orifice. A DC voltage is applied on needle which develops a charge from atmosphere and evaporated solvent, leads to production of ions in gas phase. The process of introducing the ions into the mass analyzer through differential pumping and their subsequent separation and detection are identical. This method used to improve ionization efficiency. This method is particularly useful for non-polar analyte or weakly polar analyte in normal phase.

Tandem means when two or more than two analyzer either of different type (TOF/Q) or the same type (Q/Q) configured. The quadrupole mass analyzer configured in tandem commonly called Triple Quadrupole Mass Analyzer. This configuration increases the inherent selectivity and capability of instrument exponentially. In between the two quadrupole a collision cell is used as a false quadrupole for the fragmentation of precursor ions. This cell is either hexapole or octapole not true quadrupole so this configuration is called as triple quadrupole.

6.2.3.2 Ionization Polarity

In API method either positive or negative charge ion can be generated. The type of ions generated is dependent on the charge of adduct gain or loss during ionization. e.g.: A positive DC voltage on the capillary will generate positive ion from the basic compound such as amine will favor the positively charged ions (protonated). A negative DC voltage produce negative ion from acidic compounds such as carboxylic acid will favor negatively charged ion (deprotonated).

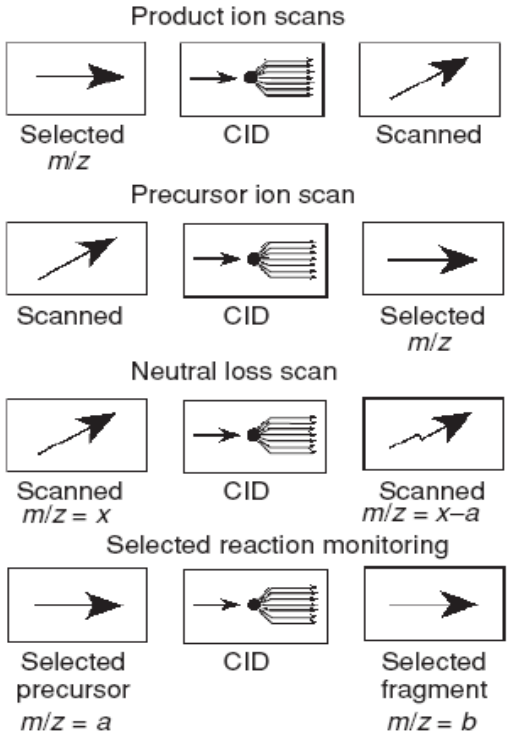
6.2.3.3 Tandem mass spectrometry

Tandem mass spectrometry is also called as mass spectrometry-mass spectrometry because the instrument contain two mass analyzer in tandem. A collision cell is placed between the two analyzers.

The basic approach of MS-MS is the measurement of mass to charge ratio of ions before and after the fragmentation of selected ions in collision cell with high-pressure gas. This collision process is known as collision induced dissociation. The tandem arrangement allows the m/z relating to the analyte to be selected at first quadrupole and m/z characteristic of daughter ions to be selected at the second

quadrupole. This arrangement provides good selectivity and sensitivity. The first quadrupole is used to select the parent ion and fragmentation occurs in the collision cell. The fragmented ion goes to second quadrupole, which select the ions of the specific reaction product only.

In this process the internal energy of analyte is increasing which induce the fragmentation this is achieved by collision activation. Collision with neutral gas molecules called collision induced dissociation (CID).



6.2.3.4 CID is a two-step process:

First collision when an ion translational energy is converted into internal energy to obtain an ion in excited state and a secondary slow unimolecular decomposition which yield various ion product from a number of competitive reaction. As a result there is increase in internal energy, leads to the fragmentation of ions. In case of CID the fragmentation of actually induced in a collision cell with

higher pressure. In principle two mass analyzers are required, one is for selecting the precursor ions from the ions generated in the ion source and other for analyzing the product ions after the collision for this approach is called tandem mass spectrometry.

6.2.4 Detector

The ions pass through the mass analyser and are then detected and transformed into causable signal by a detector. Detectors are able to generate from the incident ions an electric current that is proportional to their abundance. Detectors used in mass spectrometry were reviewed in 2005. The most common types of ion detectors are described below. (Edmond de Hoffmann 2004)

- Photographic Plate
- Faraday Cup
- Electron Multipliers

6.2.4.1 Photographic Plate

The first mass spectrometers used photographic plates located behind the analyser as detectors. Ions sharing the same m/z ratio all reach the plate at the same place and the position of the spots allows the determination of their m/z values after calibration. The darkness of the spots gives an approximate value of their relative abundance. This detector, which allows simultaneous detection over a large m/z range, has been used for many years but is obsolete today.

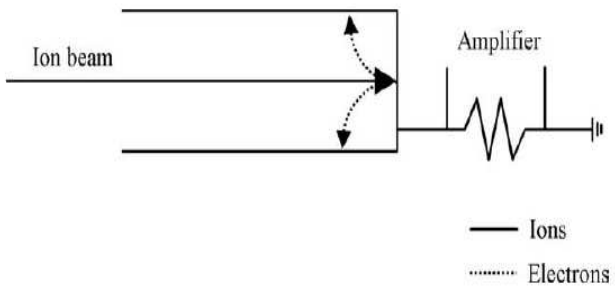
6.2.4.2 Faraday Cup

A Faraday cup is made of a metal cup or cylinder with a small orifice. It is connected to the ground through a resistor. Ions reach the inside of the cylinder and

are neutralized by either accepting or donating electrons as they strike the walls. This leads to a current through the resistor. The discharge current is then amplified and detected. It provides a measure of ion abundance.

Because the charge associated with an electron leaving the wall of the detector is identical to the arrival of a positive ion at this detector, secondary electrons that are emitted when an ion strikes the wall of the detector are an important source of errors if they are not suppressed. In consequence, the accuracy of this detector can be improved by preventing the escape of reflected ions and ejected secondary electrons. Various devices have been used to capture ions efficiently and to minimize secondary electron losses. For instance, the cup is coated with carbon because it produces few secondary ions. The shape of the cup and the use of a weak magnetic field prevent also any secondary electrons produced inside to exit.

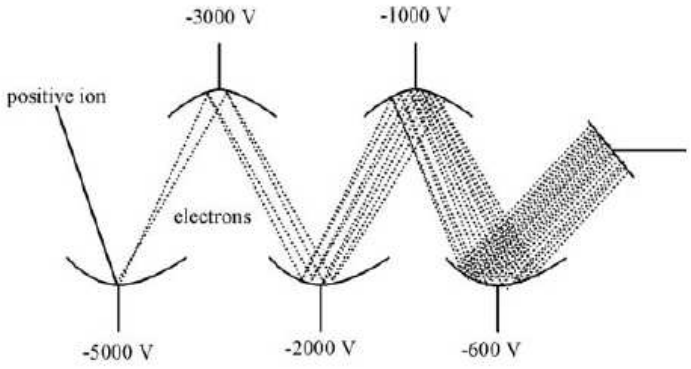
The disadvantages of this simple and robust detector are its low sensitivity and its slow response time. Indeed, the sensitivity of such detectors is limited by the noise of the amplifiers. Furthermore, this detector is not well adapted to ion currents that are not stable in the same time as during the scanning of the analyser because of its slow response time. These detectors are nevertheless very precise because the charge on the cylinder is independent of the mass, the speed and the energy of the detected ions.



6.2.4.3 Electron Multipliers

At present, the most widely used ion detector in mass spectrometry is the electron multiplier (EM). In this detector, ions from the analyser are accelerated to a high velocity in order to enhance detection efficiency. This is achieved by holding an electrode called a conversion dynode at a high potential from ±3 to ±30 kV, opposite to the charge on the detected ions. A positive or negative ion striking the conversion dynode causes the emission of several secondary particles. These secondary particles can include positive ions, negative ions, electrons and neutrals.

When positive ions strike the negative high-voltage conversion dynode, the secondary particles of interest are negative ions and electrons. When negative ions strike the positive high-voltage conversion dynode, the secondary particles of interest are positive ions. These secondary particles are converted to electrons at the first dynode. These are then amplified by a cascade effect in the electron multiplier to produce a current. The electron multipliers may be of either the discrete dynode or the continuous dynode type (channeltron, microchannel plate or microsphere plate)



The discrete dynode electron multiplier is made up of a series of 12 to 20 dynodes that have good secondary emission properties. These dynodes are held at decreasing negative potentials by a chain of resistors. The first dynode is held at a

high negative potential from−1 to−5 kV, whereas the output of the multiplier remains at ground potential. Secondary particles generated from the conversion dynode strike the first dynode surface causing an emission of secondary electrons. These electrons are then accelerated to the next dynode because it is held at a lower potential. They strike the second dynode causing the emission of more electrons. This process continues as the secondary electrons travel towards the ground potential. Thus a cascade of electrons is created and the final flow of electrons provides an electric current at the end of the electron multiplier that is then increased by conventional electronic amplification.

6.2.5 Modes of LC/MS monitoring

The first quadrupole, Q1, is operated in either a full-scan or SIM mode to select ions to pass on to the other analyzers in the system. The middle Q2 unit is flooded with a heavy inert gas, either krypton or xenon, and fragmentation is induced as the ions passed to Q2 from the first quadrupole undergo thermal collision with the higher concentration of large molecules. The final analyzer, Q3, can also be selected for either full-scan or SIM mode operation. We have two operational modes for both Q1 and Q3, providing four possible experiments that are run with a triple-quad mass spectrometer.

There are four possible modes of operation of the two analyzers:

- Q1 scan/Q3 SIM, called daughter mode or precursor scanning.
- Q1 SIM/Q3 scan, called parent mode or product scan.
- Q1 scan/Q3 scan, referred to as neutral loss scanning mode.
- Q1 SIM/Q3 SIM, referred to as multiple reaction monitoring (MRM) mode.

6.2.5.1 Daughter Mode or Precursor Scanning

SCAN/SIM mode operation lets us determine which primary fragments are related to each other. The first quadrupole is scanned over the mass range, and all fragments formed enter the collision cell and fragment to form secondary fragments. The third quadruple is parked at a specific mass/charge position and only primary fragments that break down to form this specific secondary m/z value will be detected. This common daughter ion points out interrelated primary fragments and helps us to understand which fragments are formed when a large primary fragment breaks down. In LC/MS we could store a TIC run of a peptide mixture for retention times and molecular-weight determination and then run Q3 parked at mass 79 and lay this chromatogram over the TIC to detect phosphorylated peptides present in the mixture.

6.2.5.2 Parent Mode or Product Scan

SIM/scan operations parks the first quadrupole analyzer at a specific mass, allowing only a single primary fragment to enter the collision cell where it fragments into secondary ions. The final quadrupole is run in full-scan mode, detecting all secondary product fragments formed from this single primary parent, again providing structural information by showing its breakdown products. This mode is commonly used in LC/MS to examine a molecular ion for its mass spectrometer fragments to provide structural identification.

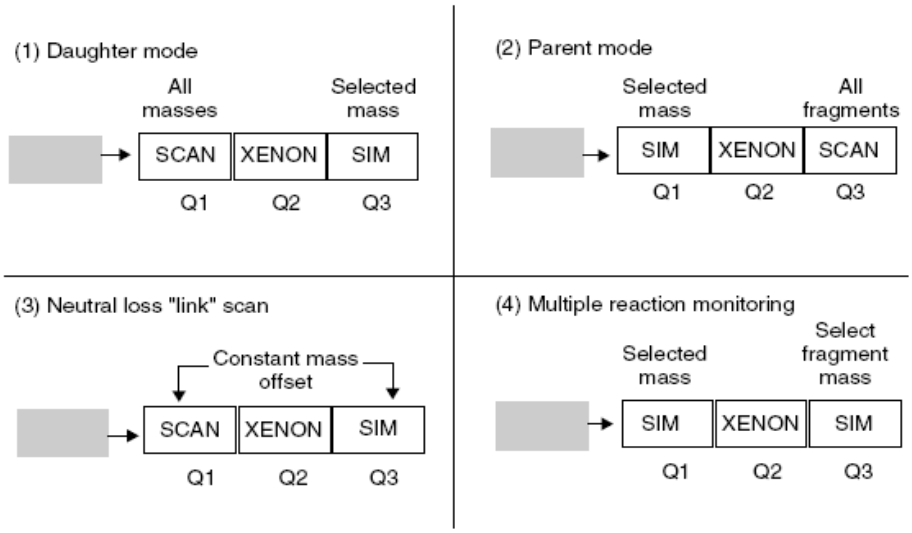
6.2.5.3 Neutral Loss Scanning Mode

Scan/scan operation, or neutral loss mode, is a little more complicated since both analyzer quadrupoles will be scanned at the same time but with a preset mass offset.

When a primary fragment undergoes further fragmentation, it breaks into two pieces, a charged secondary fragment and a neutral molecule. What we are detecting in this mode are primaries that lose the same neutral molecule, such as carbon monoxide, water, or a vinyl compound, and therefore may be breaking down by the same fragmentation mode. The molecular mass of our suspected "neutral loss" is the value we assign to our scan offset between the two scanning quadrupole analyzers. All primary fragments separated in the first analyzer enter the collision cell and fragment. Only secondary fragments whose mass is exactly the neutral loss smaller than their primary fragment are detected after the final quadrupole and show up in the display of the scanning chromatogram. Any primary fragment that breaks down by forming a neutral molecule that has a mass different from the offset mass will not be selected by the second analyzer and enter the detector.

6.2.5.4 Multiple Reaction Monitoring (MRM) Mode

SIM/SIM operation is designed to analyze specific components of very impure mixtures definitively without having to purify them completely. They can be detected at a very high sensitivity since both analyzers are parked at different specific single m/z values, and a greater number of scans can be summed in determining their position. Nature makes very complex mixtures that cannot always be separated completely either though extractions or by chromatography. We examine a chromatographic peak in which we expect a specific compound to appear by using the first quadrupole to separate a primary fragment characteristic of the compound of interest, pass it into the collision cell, and use the final quadrupole to identify it by looking for only one of its specific daughter ions. (Edmond and Hoffmann 2004).



We can identify and quantitate each targeted compound in a mixture, even if the chromatographic peaks that contain them are contaminated. For each compound to be analyzed, we select an individual primary and secondary fragment on a time basis, in step with their expected chromatographic retention time.

LITERATURE REVIEW

- **Mary Ellen Sweeney, Rebecca R. Johnson, Expert Opinion, Drug evaluation, Ezetimibe: an update on the mechanism of action, pharmacokinetics and recent clinical trials, 2007, (441-450).**

Elevated serum cholesterol is a known risk factor for the development of coronary artery disease. Circulating cholesterol is a product of both cholesterol absorption from the gut and cellular cholesterol production. Ezetimibe is a novel cholesterol-lowering drug that acts at the brush border of the small intestine. Recent studies have further identified the molecular target as the Niemann-Pick C1-like transporter. Ezetimibe blocks the absorption of dietary and biliray sterols resulting in intracellular cholesterol depletion. Clinical studies have demonstrated beneficial improvements in the lipid profile with Ezetimibe as monotherapy, but dramatic effects are seen when Ezetimibe is combined with other lipid-lowering drugs, particularly 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins). Combination studies of Ezetimibe with statins, bile acid sequestrants, fenofibrate and niacin all demonstrate significant total and low density lipoprotein cholesterol lowering. An excellent safety and tolerability profile combined with once-daily dosing make this attractive adjunct therapy for the treatment of hypercholesterolemia.

- **Unnam Seshachalam, Chandrasekhar B. Kothapally, HPLC Analysis for Simultaneous Determination of Atorvastatin and Ezetimibe in Pharmaceutical Formulations, Journal of Liquid Chromatography and Related Technologies, (2008) 32:5, 714-721.**

A simple, isocratic, and sensitive reverse phase high performance liquid chromatographic(RP-LC) method has been developed, for the first time, for quantitative determination of Atorvastatin and Ezetimibe in pharmaceutical formulations, Atorvastatin and Ezetimibe were chromatographed using 0.01 M ammonium acetate buffer (pH: 3.0): Acetonitrile (50:50 v/v) as mobile phase. The detection was monitored at 254nm. The retention time of ezetimibe and Atorvastatin were 15.50 and 19.30 respectively. The linearity of the method was studied over the concentration range of 4-400 µg/mL for Atorvastatin and 5-500 µg/mL for Ezetimibe. The limit of detection for Atorvastatin and Ezetimibe were found as 1.25µg/mL and 1.48µg/mL, respectively. The proposed method was applied for the quantitative determination of Atorvastatin and Ezetimibe in commercial combination formulations.

- **Stefan Ostwald, Eberhard Scheuch, Ingolf Cascorbi, Werner Siegmund, A LC-MS/MS method to quantify the novel cholesterol lowering drug Ezetimibe in human serum, urine and feces in healthy subjects genotyped for SLCO1B1, Journal of Chromatography B, 830(2006), 143-150.**

Ezetimibe is a novel cholesterol lowering drug which disposition is not fully understood in man. We developed a selective and high-sensitive assay to measure serum concentration-time profiles, renal and fecal elimination of ezetimibe in pharmacokinetic studies. Ezetimibe glucuronide, the major metabolite of ezetimibe was demonstrated by enzymatic degradation to the parent compound. Ezetimibe was measured after extraction with tert-butyl ether using 4-hydroxychalcone as internal standard and liquid chromatography coupled with an APCI interface with tandem mass spectrometry for detection. The chromatography (column XTerra MS C18, 2.1

mm x 100mm, particle size 3.5µm) was done isocratically with Acetonitrile: water: 60:40, flow rate 200µL/min. The MS/MS analysis was performed in the negative ion mode. The validation ranges for Ezetimibe were as follow serum 0.00001-0.015µg/mL and 0.001-0.2 µg/mL; urine and fecal homogenate 0.025-10 µg/mL and 0.1-20 µg/mL, respectively. The assay was successfully applied to measure ezetimibe disposition in two subjects genotyped for the hepatic uptake transporter SLCO1B1.

- **Oliveira P. R., Brum Junior L., Fronza M., Bernardi L. S., Masiero M. K., Dalmora S. L., Development and Validation of a Liquid chromatography-Tandem Mass Spectrometry Method for the determination of Ezetimibe in Human Plasma and Pharmaceutical Formulations, Chromatographia 2006, 63.**

An Analytical method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed and validated for the determination of ezetimibe in human plasma. Ezetimibe and Etroricoxib (Internal Standard) were extracted from the plasma by liquid–liquid extraction and separated on a C18 analytical column (50 x 3.0 mm ID) with Acetonitrile: water: 85:15 as mobile phase. Detection was carried out by positive electro spray ionization in multiple-reaction monitoring (MRM) mode. The chromatographic separation was obtained within 2.0 min and was linear in the concentration range of 0.25-20ng/mL for free ezetimibe and of 1-300ng/mL for total ezetimibe. The mean extraction recoveries for free ezetimibe and total ezetimibe from plasma were 96.14 and 64.11% respectively. Method validation investigated parameters such as linearity, precision accuracy, specificity and stability, giving results within the acceptable range. The proposed method was successfully applied to the quantification of ezetimibe and its

glucuronide in human plasma to support clinical and pharmacokinetics studies. Moreover, the method was used for the quality control analysis of pharmaceutical dosage forms.

- **Hossein Danafar, Mehrdad Hamidi, a Rapid and Sensitive LC-MS Method for determination of Ezetimibe concentration in Human Plasma: Application to a Bioequivalence Study, Chromatographia (2013) 76:1667-1675.**

A Selective and highly sensitive high performance liquid chromatography-electrospray ionization mass spectrometry method has been developed for determination of ezetimibe in human plasma. Ezetimibe was extracted from plasma with ethyl acetate followed by evaporation of the organic layer and then, reconstitution of the residue in mobile phase before injection to chromatograph. The mobile phase constituted of Acetonitrile-ammonium acetate (10mM. pH 3.0), 75:25. An aliquot of 10µL was chromatographically analyzed on a pre-packed Zorabax XDB-ODS C18 column (2.1 x 100mm, 3.5 micron). Detection of analytes was achieved by mass spectrometry with atmospheric pressure chemical ionization (APCI) interface in the negative ion mode operated under the multiple-reaction monitoring mode (MRM). Standard curve were linear over the wide ezetimibe concentration range of 0.005-30.0ng/mL with acceptable accuracy and precision. The limit of detection was 0.02ng/mL. The validated LC-APCI-MS method has been successfully throughout a bioequivalence study on an ezetimibe generic product in 24 healthy male volunteers.

- **Shuijun Li, Gangyi Liu, Jingying Jia, Xiaochuan Li, Chen Yu, Liquid Chromatography-Negative ion Electrospray Tandem Mass Spectrometry for the Quantification of Ezetimibe in Human Plasma, Journal Of Pharmaceutical And Biomedical Analysis, 40 (2006)., 987-992.**

A simple, reliable and sensitive liquid-liquid chromatography-tandem spectrometry method (LC-MS/MS) was developed and validated for quantification of free and total ezetimibe in human plasma. The analyte and internal standard (Ezetimibe 13C6) were extracted by liquid-liquid extraction with methyl tert-butyl ether. The reversed-phase chromatographic separation was performed on a Capcell C18 column, and the plasma extract was eluted with a gradient consisting of Acetonitrile and 5mM ammonium acetate. The analyte was detected using negative ionization by multiple-reaction monitoring mode. The mass pair of 408.5 ⟶ 270.8 and m/z 414.5 ⟶ 276.8 were used to detect ezetimibe and internal standard, respectively. The assay exhibited linear ranges from 0.02 to 20ng/mL for free ezetimibe and 0.25 to 250ng/mL for total ezetimibe in human plasma. Acceptable precision and accuracy were obtained for concentration of the calibration standard and quality control. The validated method was successfully used to analyze human plasma samples for application in a pharmacokinetic study.

- **Sistla R., Tata V. S. S. K., Kashyap Y. V., Chandrasekhar D., Diwan P. V., Development and validation of a reversed phase HPLC method for the determination of ezetimibe in Pharmaceutical dosage forms, Journal Of Pharmaceutical And Biomedical Analysis, 39 (2005), 517-522.**

Ezetimibe belongs to a group of selective and very effective 2-azetidone cholesterol absorption inhibitors that act on the level of cholesterol entry into

enterocytes. A rapid, specific reversed-phase HPLC method has been developed for assaying ezetimibe in pharmaceutical dosage forms. The assay involved an isocratic elution of ezetimibe in Kromasil 100 C18 column using a mobile phase composition of water (pH 6.8, 0.05%, 1-heptane sulfonic acid) and Acetonitrile (30:70). The flow rate was 0.5mL/min and the analyte monitored at 232 nm. The method was successfully applied to estimate the amount of ezetimibe in tablets.

DRUG PROFILE

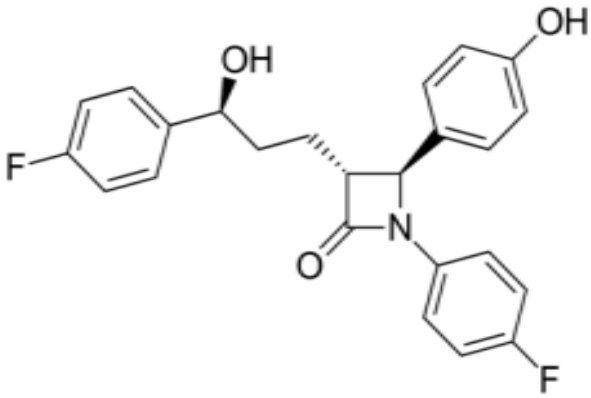
DRUG NAME

Ezetimibe

CHEMICAL NAME

((3R,4S)-1-(4-Fluorophenyl)-3-[(S)-3-(4-Fluorophenyl)-3-hydropropyl]-4-(4hydrophenyl) azetidine-2-one)

MOLECULAR STRUCTURE



MOLECULAR FORMULA

$C_{24}H_{21}F_2NO_3$

MOLECULAR MASS

409.43 g/mol

PROPERTIES

- Ezetimibe is an odorless white crystalline powder,
- It having a bitter taste.
- It is soluble in dilute acids, methanol and chloroform.
- It is practically insoluble in water.

CATEGORY

Anti-hyperlipidaemic

CLINICAL PHARMACOLOGY

Ezetimibe, a novel drug that acts by inhibiting intestinal absorption of cholesterol and phytosterols. It interferes with a specific CH transport protein NPC1L1 in the intestinal mucosa and reduce absorption of both dietary and biliray CH.

MECHANISM OF ACTION

The mechanism of action is distinct from other potent lipid- lowering drug that acts in the gut. Ezetimibe is a potent inhibitor of cholesterol and phytosterols absorption in the small intestine, where both dietary and biliary cholesterol are available for absorption. However, its action is unique in that does not affect cholesterol micelle formation or increase bile acid secretion. It does not alter fat-soluble vitamin and nutrient absorption.

PHARMACOKINETICS

1. ABSORBTION

Peak plasma concentration of total Ezetimibe (free Ezetimibe + Ezetimibe Glucuronide) 1-2 Hr. Concomitant food administration has no effect on the rate or extent of oral bioavailability. Ingestion of a high fat meal significantly increased C_{max} (by 44%) and decreased T_{max} with no net change in AUC. This change was not considered clinically significant and can be taken without regard to meals or fat content.

2. DISTRIBUTION

Highly bound to plasma protein (>93%) and not affected by chronic renal disease or moderate chronic liver failure.

3. METABOLISM

Extensive presystemic metabolism via intestinal uridine 5'diposphate-glucuronosyl transferase enzyme to the active glucuronyl metabolite (Ezetimibe metabolite).Ezetimibe and its conjugate and then transported via the portal vessels to the liver where they undergo additional glucuronidation and subsequent bilary secretion into the intestine. Ezetimibe-glucuronide accounts for the 80-90% of drug in plasma. Plasma concentration time profiles exhibit multiple peaks, suggesting enterohepatic recirculation. The rate and extent can be affected by either intestinal or hepatic secretion via P-glycoprotein and multi drug resistance- associated protein 2.

Ezetimibe is neither an inhibitor nor an inducer and only a minor substrate of common CYP iso-enzymes at clinically relevant doses.

4. EXCRETION

Bilary secretion into the intestine and subsequent excretion in the feces (78%) and urine (11%). The parent drug is the major component in feces and ezetimibe-glucuronide in urine. Following repeated dose administration, the estimated terminal elimination half-life is 16-31 hrs. Consistent with the elimination half life, a two folds accumulation is observed with repeated once-daily administration.

AIM AND PLAN OF WORK

Studies to measure bioavailability and /or establish bioequivalence of product are important elements in support of orally administered Drug products in Investigational New Drug applications (INDs), New DRUG Applications (NDAs), Abbreviated New Drug application (ANDAs) and their supplements. The systemic exposure profile determined during clinical trial in IND period can serve as benchmark for subsequent BE studies.

Mathematic analysis of plasma level v/s time curve permit estimation of half lives, absorption, excretion rates, extent of absorption (area under curve) and constants that are useful in determining the fate of a given Drug in an organism. Comparative bioavailability studies permit judgement as to the bioequivalence of Drugs. These determinations may in turn lead to important decision related to Drug product selection by pharmacists.

Until recently, bio-availability (rate and extent of absorption of medicaments from drug delivery systems) of drugs was note emphasized. It was more or less assumed that if the physical and chemical integrities of a drug product were assured pharmacologic performance would be observed. It is now recognize that formulation factors can influence the biologic availability of a medicament from a dosage unit in mammalian systems. Consequently, it has become common practice to establish bio-availability by measurement of blood levels of drugs following administration of dosage forms.

However it should be noted that neither bioavailability nor bioequivalence data could be generated without analytical methodology to accurate measure Drug in biological fluids.

Methods of measuring drugs in biological media are increasingly important problems related to bio-availability and bio-equivalence, new drug development, drug abuse, clinical pharmacokinetics and drug research are highly dependent on accurately measured drugs in biological fluids. Bioavailability & bioequivalence, new Drug development, Drug abuse, clinical pharmacokinetics & Drug research are highly dependent on accurately measuring Drugs in biological fluids.

For estimation of Drugs and its in vivo active metabolite present in biological fluid LC/MS/MS method is considered to be most suitable since it is a powerful and rugged method. It is also extremely specific, accurate, sensitive and rapid when compared to the other methods.

Present experiment is to develop & validate a selective and sensitive method for the quantitative determination of Ezetimibe in human plasma using LC-MS/MS technique.

PLAN OF THE PRESENT STUDY IS AS FOLLOWS:

1. Optimization of chromatographic conditions was proposed to be developed and optimized.

- Selection of wavelength,
- Selection of initial separation conditions,
- Nature of the stationary phase,

- Nature of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate),
- Sensitivity

2. The developed method were also proposed to be validated using the various validation parameters such as,

- System Suitability Test
- Selectivity
- Carry over
- Matrix Effect
- Precision and Accuracy
- Recovery
- Dilution integrity
- Ruggedness
- Reinjection Reproducibility
- Hemolysis Effect
- Reagent Stability
- Stabilities.

MATERIALS AND METHODS

DRUGS USED

Standards	Batch No./ Lot No.	Manufacture	Retest Date/ Date of Expiry
Ezetimibe	F028D0	USP	Current Lot
Ezetimibe D4	CRC-0213-094	Clearsynth	08May2019
Ezetimibe Phenoxy Glucuronide	1424-095A8	TLC	14Jul2020

CHEMICALS AND REAGENTS USED

Materials and Reagent	Grade	Make/Suppliers
Sodium Acetate Trihydrate	AR	CDH
Sodium Hydroxide	AR	Merck
β-Glucuronidase	N/AP	Sigma Aldrich
Glacial Acetic Acid	AR	Merck
Diethyl Ether	AR	Spectrochem
Acetonitrile	LCMS	JT Baker
L-Ascorbic Acid	AR	SDFCL
Methanol	LCMS	JT Baker
Ammonium Acetate	AR	Sigma Aldrich
Water	Milli-Q	Millipore
K2EDTA Plasma	N/AP	Rajya Lakshmi Blood Bank

INSTRUMENT USED

Name	Model / Brand	Make
Multi Tube Vortexer	1401002	Tarson
Vortex Shaker	SPINIX	Tarson
Analytical Balance	MSA225S-100-DA	Sartorius
Micro Balance	MSA2.7S-000-DM	Sartorius
pH Meter	Star A211	Thermo
Centrifuge	5810 R	Eppendorf
Deep freezer (-65 ±10ºC)	MDF-U74V-PE	Panasonic
Deep freezer (-20 ±5ºC)	MDF-U731M-PE	Panasonic
Refrigerator	MPR-514-PE	Panasonic
Refrigerator	FKG 371	Vestfrost
Auto Pipette	RESEARCH PLUS	Eppendorf
Multipette	M4	Eppendorf
Milli Q water system	ADVANTAGE A10	Millipore
Ultra Sonicator	Ultrasons-HD	J.P. Selecta
Nitrogen Evaporator	Turbo Vap lv	Biotage

PREPARATION OF SOLUTIONS

1. Ammonium Acetate Buffer (pH 4.0)

Weighed approximately 385.400 mg of ammonium acetate and transfered into a 1000.000 mL reagent bottle containing 1000.000 mL of water. Adjusted the pH to 4.0 using glacial acetic acid. Filtered, sonicate and labeled.

2. Diluent (Methanol : Water :: 80:20)

Transferred 800.000 mL of Methanol into a regent bottle and added 200.000 mL of Water mixed well and labeled.

3. 0.5 M Sodium Acetate Buffer (pH 5.0)

Weighed approximately 6.804 g of sodium acetate trihydrate and transferred it into a 100.000 mL reagent bottle containing 100.000 mL of water, mixed well and adjusted the pH to 5.0 using glacial acetic acid and labeled.

4. 1.00% L-ascorbic Acid

This solution is used as a diluent for the preparation of β-Glucuronide enzyme solution.

Weighed approximately about 1.000 g of L-Ascorbic acid and transferred it into a 100.000 mL reagent bottle containing 100.000 mL of water, mixed well and labeled.

5. β-Glucuronidase Enzyme Solution(20000 IU/mL)

This solution is used for hydrolysis enzymatic reaction.

Prepared approximately about 20000 IU/mL of β-Glucuronidase Enzyme solution using 1.00% L-ascorbic Acid as a diluent, vortexed to mix and labeled.

Note: β-Glucuronidase Enzyme stock concentration may vary from lot to lot.

For Example: If stock concentration is 100000 IU/mL, take 2.000 mL enzyme from the stock and dilute it to 10.000 mL with 1.00 % L- Ascorbic Acid.

6. 0.1 N Sodium Hydroxide

Weighed approximately about 400.000 mg of sodium hydroxide and transferred it into a 100.000 mL reagent bottle containing 100.000 mL of water, mixed well and labeled.

7. Mobile Phase (Acetonitrile: Buffer:: 75:25)

Transferred 750.000 mL of Acetonitrile into a 1000.000 mL reagent bottle and added 250.000 mL of 5mM Ammonium acetate buffer (pH 4.0) and mixed well, sonicate and labeled.

8. Rinsing Solution

Acetonitrile shall be used as rinsing solution.

PREPARATION OF EZETIMIBE CALIBRATION CURVE STANDARDS

1. Stock Solution Preparation of Ezetimibe

Weighed accurately about 2.0198 mg of Ezetimibe working standard and transferred into 2.000 mL volumetric flask containing methanol and diluted up to the mark with the same. Calculate the actual concentration using amount weighed and purity of Ezetimibe.

$$\text{Stock conc.} = \frac{\text{Wt. taken, x molecular wt. x assay percent x 1000}}{\text{Total volume, (ml) x formula weight x 100}}$$

Weight taken (mg)	Formula Weight	Molecular Weight	Assay %	Total volume, (mL)	Conc. ng/mL
2.0198	409.43	409.43	99.70	2.000	1006870.300

2. Preparation of Ezetimibe Intermediate Solution1 (100687.030 ng/mL)

Pipette out 1.000 mL of Ezetimibe stock solution into 10.000 mL volumetric flask and diluted up to the mark with the diluent.

3. Preparation of Ezetimibe Intermediate Solution2 (10068.703 ng/mL)

Pipette out 1.000 mL of intermediate stock solution1 into 10.000 mL volumetric flask and diluted up to the mark with the diluent.

4. Preparation of Ezetimibe Intermediate Solution3 (1006.870 ng/mL)

Pipette out 1.000 mL of intermediate stock solution2 into 10.000 mL volumetric flask and diluted up to the mark with the diluent.

5. Preparation of Working Solution of Ezetimibe

Working solution of different concentration of Ezetimibe was prepared using Volumetric Flask using different volumes of above intermediate solutions as described in following Table No. 1, for calibration curve standards. These intermediate solutions was diluted up to the mark with the diluent.

Table No. 1 Preparation of Working Solution for CC Standards

Intermediate ID	Volume of Intermediate (mL)	Total Volume (mL)	Working Solution Concentration of Ezetimibe (ng/mL)	Working Solution ID
Intermediate Solution3	0.250	10.000	25.000	WCS1
Intermediate Solution3	0.500	10.000	50.000	WCS2
Intermediate Solution2	0.625	10.000	625.000	WCS3
Intermediate Solution2	1.250	10.000	1250.000	WCS4
Intermediate Solution2	2.500	10.000	2500.000	WCS5
Intermediate Solution1	0.500	10.000	5000.000	WCS6
Intermediate Solution1	0.750	10.000	7500.000	WCS7
Intermediate Solution1	1.000	10.000	10000.000	WCS8
Intermediate Solution1	1.250	10.000	12500.000	WCS9
Intermediate Solution1	0.625	10.000	6250.000	WSSS

6. Preparation of Calibration Curve Standards

Calibration curve standards shall consist of blank plasma, zero blank and non-zero calibration curve standards covering the expected range of concentrations of analyte. Stock dilutions shall be used for the preparation of different calibration curve standards and resulted concentrations of Ezetimibe in spiked plasma for different calibration curve standards is as described in Table No. 2.

Note: For Blank plasma and Zero Blank samples, spike 2.00% of Diluent in place of working solution.

Table No. 2 Preparation of Calibration Curve Standards

Working Solution ID	Volume of Blank Plasma (mL)	Working Solution Volume (mL)	Final Volume (mL)	Final Concentration of Ezetimibe (ng/mL)	Calibration Curve Standards ID
WCS1	9.800	0.200	10.000	0.500	CS1
WCS2	9.800	0.200	10.000	1.000	CS2
WCS3	9.800	0.200	10.000	12.500	CS3
WCS4	9.800	0.200	10.000	25.000	CS4
WCS5	9.800	0.200	10.000	50.000	CS5
WCS6	9.800	0.200	10.000	100.000	CS6
WCS7	9.800	0.200	10.000	150.000	CS7
WCS8	9.800	0.200	10.000	200.000	CS8
WCS9	9.800	0.200	10.000	250.000	CS9
WSSS	9.800	0.200	10.000	125.000	SSS

PREPARATION OF EZETIMIBE QUALITY CONTROL SAMPLES

1. Stock Solution Preparation of Ezetimibe

Weighed accurately about 2.0792 mg of Ezetimibe working standard and transferred into 2.000 mL volumetric flask containing methanol and diluted up to the mark with the same. Calculate the actual concentration using amount weighed and purity of Ezetimibe.

$$\text{Stock conc.} = \frac{\text{Wt. taken, x molecular wt. x assay percent x 1000}}{\text{Total volume, (ml) x formula weight x 100}}$$

Weight taken (mg)	Formula Weight	Molecular Weight	Assay %	Total volume, (mL)	Conc. ng/mL
2.0792	409.43	409.43	99.70	2.000	1036481.200

2. Stock Solution Preparation of Ezetimibe Phenoxy Glucuronide

Weighed accurately about 2.9887 mg of Ezetimibe Phenoxy Glucuronide (Equivalent to 2.000 mg of Ezetimibe) and transferred into 2.000 mL volumetric flask containing methanol and diluted up to the mark with the same. Calculate the actual concentration using amount weighed and purity of Ezetimibe.

$$\text{Stock conc.} = \frac{\text{Wt. taken, x molecular wt. x assay percent x 1000}}{\text{Total volume, (ml) x formula weight x 100}}$$

Weight taken (mg)	Formula Weight	Molecular Weight	Assay %	Total volume, (mL)	Conc. ng/mL
2.9887	585.56	409.43	97.90	2.000	1022923.790

3. Preparation of Ezetimibe and Ezetimibe Phenoxy Glucuronide Intermediate Solution4 (102563.527 ng/mL)

Pipette out 0.200 mL of Ezetimibe stock solution and 0.800 mL of Ezetimibe Phenoxy Glucuronide stock solution into 10.000 mL volumetric flask and dilute up to the mark with the diluent.

4. Preparation of Ezetimibe and Ezetimibe Phenoxy Glucuronide Intermediate Solution5 (10256.353 ng/mL)

Pipette out 1.000 mL of intermediate stock solution4 into 10.000 mL volumetric flask and dilute upto the mark with the diluent.

5. Preparation of Ezetimibe and Ezetimibe Phenoxy Glucuronide Intermediate Solution6 (1025.635 ng/mL)

Pipette out 1.000 mL of intermediate stock solution5 into 10.000 mL volumetric flask and dilute upto the mark with the diluent.

Note: Subsequent intermediate dilutions shall be prepared from previous intermediate instead of respective stock solution.

6. Preparation of Working Solution of Ezetimibe

Working solution of different concentration of Ezetimibe shall be prepared using suitable container/Volumetric Flask using different volumes of above intermediate solutions as described in following Table No. 3, for quality control samples. These intermediate solutions shall be diluted up to the mark with the diluent.

Table No. 3 Preparation of Working Solution for QC Samples

Intermediate ID	Volume of Intermediate (mL)	Total Volume (mL)	Working Solution Concentration of Ezetimibe (ng/mL)	Working Solution ID
Intermediate Solution6	0.250	10.000	25.000	WLLOQQC
Intermediate Solution6	0.600	10.000	60.000	WLQC
Intermediate Solution5	2.000	10.000	2000.000	WMQC1
Intermediate Solution5	5.500	10.000	5500.000	WMQC
Intermediate Solution4	1.000	10.000	10000.000	WHQC
Intermediate Solution4	4.000	10.000	40000.000	WDIQC

Diluent: Methanol: Water (80: 20 v/v)

7. Preparation of Quality Control Samples

Quality control samples shall be prepared in screened blank/ pooled plasma with stock dilutions of known concentrations. The QC samples shall be prepared in

replicates at each different concentration levels i.e. LLOQ QC, LQC, MQC1, MQC, HQC, and DIQC). Stock dilutions shall be used for the preparation of different concentration levels of QC and resulted concentrations of Ezetimibe in spiked plasma for different QC samples as described in Table no. 4.

TABLE No. 4 Preparation of Quality Control Samples

Working Solution ID	Volume of Blank Plasma (mL)	Working Solution Volume (mL)	Final Volume (mL)	Final Concentration of Ezetimibe (ng/mL)	Quality Control ID
WLLOQQC	9.800	0.200	10.000	0.500	LLOQQC
WLQC	9.800	0.200	10.000	1.200	LQC
WMQC1	9.800	0.200	10.000	40.000	MQC1
WMQC	9.800	0.200	10.000	110.000	MQC
WHQC	9.800	0.200	10.000	200.000	HQC
WDIQC	9.800	0.200	10.000	800.000	DIQC

Note:

1. QC samples shall be prepared containing both Ezetimibe Phenoxy Glucuronide and Ezetimibe to mimic the study sample analysis requirement as the study samples will contain both Ezetimibe Phenoxy Glucuronide and Ezetimibe. The QC samples shall be prepared in the proportion of 80: 20 where 80% of Ezetimibe Phenoxy Glucuronide and 20% of Ezetimibe.

2. As spiking solutions of calibration curve standard are prepared using Ezetimibe only and spiking solutions of quality control samples were prepared using Ezetimibe and Ezetimibe Phenoxy Glucuronide, spiking solution check experiment shall be performed in extracted medium (using spiking solution spiked into water (Milli Q/HPLC Grade).

PREPARATION OF EZETIMIBE D4

1. Preparation of Ezetimibe D4 Internal Standard Stock Solution

Weighed about 2.000 mg of Ezetimibe-D4 working standard and transferred into 2.000 mL volumetric flask containing methanol and diluted up to the mark with the same. Calculate the actual concentration using amount weighed and purity.

2. Preparation of Ezetimibe-D4 Internal Standard Intermediate Solution (10000.000 ng/mL)

Pipette out 0.100 mL of Ezetimibe-D4 stock solution into 10.000 mL volumetric flask and dilute up to the mark with the diluent.

3. Preparation of Ezetimibe-D4 Internal Standard Working Solution (50.000 ng/mL)

Pipette out 0.500 mL of Ezetimibe-D4 internal standard intermediate solution into 100.000 mL volumetric flask and dilute up to the mark with the diluent.

PREPARATION OF EXTRACTED AQUEOUS LINEARITY SOLUTION

The working solution of calibration curve standards and quality control samples shall be spiked in water and extracted as per extraction procedure.

PREPARATION OF SYSTEM SUITABILITY SOLUTION (SSS)

1. Aqueous SSS

Pipette out 4.000 µL of working solution WSSS- (6250.000 ng/mL for Ezetimibe), add 50.000 µL of Internal Standard Working Solution WIS- (50.000 ng/mL each of Ezetimibe D4) and add 146.000 µL of mobile phase and vortex it.

2. Extracted SSS

SSS sample will be processed as per extraction procedure and will be used as extracted system suitability sample for system suitability test.

Note: Dilutions can be adjusted in proportion during solution preparation and spiking however final concentration should remain same.

EVALUATION

The standard curves were constructed from the peak area ratio (P.A.R.) of drug / IS using linear regression $y = ax + b$ with $1/x^2$ weighing.

Drug concentrations (ng/mL) for QCs in a batch calculated by interpolating the peak area ratios from the corresponding standard curve.The measured peak area ratios of the QC samples were converted into concentration.

Using the following equation,

$$\text{Drug concentration} = \frac{\text{P.A.R.(Drug / IS)} - b}{a}$$

Where

a = Slope of the corresponding standard curve,

b = Intercept of the corresponding standard curve.

The concentrations were reported in nanogram per milliliter plasma.

TUNING OF DRUG AND INTERNAL STANDARD

For drug and IS the tunning was done manually using Methanol: water (50:50) with syringe needle.

First scanning was performed for Q1 to get mass of the parent ion. Then this ion goes to Q2 where fragmentation takes place by collision energy leads to the formation of daughter ions. This daughter ion was scanned in Q3.

Formation of ions and traveling from one quadrapole to another all activities performed by setting different potential. C.E, C.X.P, E.P and DP.

INSTRUMENT SETTING :(API-4000)

1. For Ezetimibe

Scan Type	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter			
					DP	EP	CE	CXP
MRM	Negative	408.100	271.000	200	-57	-10	-22	-10

2. For Ezetimibe D4

Scan Type	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter			
					DP	EP	CE	CXP
MRM	Negative	412.100	275.000	200	-55	-10	-22	-15

INSTRUMENT PARAMETERS

CUR	CAD	GS1	GS2	TEM	IS
20	5	50	50	500	-4500

For this particular project tuning was performed in negative mode for analyte and for internal standard.

PRETREATMENT OF BIOLOGICAL SAMPLE

In bio-analysis the method development step additionally require the extraction trial in order to recover the analyte and internal standard from highly complicated biological matrix one should have knowledge about the nature of the Drug, its molecular weight, polarity, pKa, ionic character and the solubility parameter.

Selection of internal standard should be on the basis of structural similarity, physicochemical properties related to the analyte to be quantified.

In initial stages of method development our focus is on achieving our LLOQ level with precision and accuracy and checking for interference at the retention time of analyte and internal standard.

Method development trials involve a lot of exercises based on chemistry of molecule and efficiency of our extraction procedure in order to achieve good results. Optimization of mobile phase, buffer, and column are essential part of method development.

The peak shape, retention time of analyte and internal standard, column, flow rate are optimized by making aqueous solution of drug and internal standard in a set of different mobile phase compositions.

SAMPLE PROCESSING

Prior to starting the extraction procedure, ensure that the required reagent have been prepared and are within the usage period.

Retrieve one CC standard and/or QC samples and/ or spiked samples from the deep freezer and allow to thaw at room temperature.

Vortex the thawed samples to ensure complete mixing of content.

Extract Plasma and Aqueous samples by Liquid-Liquid extraction method as described below:

- Place an appropriate number of RIA vial in a rack.
- Add 50.000 µL of WIS (50.000 ng/mL) into each tube except plasma blank.
- Aliquot 0.200 mL plasma sample into previously labeled tube and vortex it.
- Add 250.000 µL of 0.5 M sodium acetate (pH 5.0) and vortex it.
- Add 50.000 µL of enzyme solution (20000 IU/mL) and vortex it.
- Incubate the samples in water bath for 30 min at 50ºC.
- Add 250.000 µL of 0.1 N sodium hydroxide and vortex.
- Add 3.000 mL of Diethyl ether.
- Vortex the sample at 2500 RPM for 5 min in multi-tube vortexer.
- Centrifuge at 10ºC for 5 min at 4000 RPM.
- Flash Freeze the plasma layer and transfer the organic layer in to pre-labeled tubes.
- Evaporate till dryness under nitrogen steam at 40ºC
- Reconstitute with 200.000 µL of Mobile phase and vortex.
- Transfer the contents to Autosampler vials.

METHOD DEVELOPMENT TRAILS

Table No. 5 Method Development Trails

Run No	Extraction Procedure	Chromatographic Conditions	Change Mode	Observations
1	SPE Method with Oasis ® HLB 3cc 60mg SPE cartilage	Column:Hypurity C18,50X4.6 Flow rate:0.6ml Inj Volm:10µl RT :5Min MP:ACN:2mM Amm.Acetate(70:30)	NOT APPLICABLE	Area of drug and analyte & IS found to be satisfactory.
2	SPE Method with Oasis ® HLB 3cc 60mg SPE cartilage	Column:Hypurity C18,50X4.6 Flow rate:0.6ml Inj Volm:5µl RT :2.2Min MP:ACN:2mM Amm.Acetate(90:10)	100µl 5% Ortho phosphoric acid was added during sample processing and run time changed 5min to 2.2min	Recovery found to be more & chromatography found to be satisfactory.
3	LLE with tertiary butyl methyl ether'	Column:Hypurity C18, 50X4.6 Flow rate:0.6ml Inj Volm:10µl RT :5Min MP: MeOH:2mM Ammonium Acetate(70:30)	Extraction metod was changed from SPE to LLE	Results are within the acceptable criteria. but poor chromatograms was Observed.
4	LLE with ethyl acetate	Column: Hypurity C18, 50X4.6 Flow rate:0.6ml Inj Volm:10µl RT :5Min MP: MeOH:2mM Ammonium Acetate(70:30)	Tertiary butyl methyl ether is replaced with ethyl acetate	Area of drug and analyte & IS found to be satisfactory. And Chromatography need to develop.
5	LLE with Diethyl ether	Column:Gemini, C18,150X Flow rate:0.5ml Inj Volm:10µl RT :5Min MP:ACN:5mM Amm.Acetate(75:25)	Ethyl acetate is replaced with diethyl ether	Recover of analyte and IS is satisfactory, Chromatography is satisfactory and fixed as final extraction method

SELECTED CHROMATOGRAPHIC CONDITION

Column	:	Gemini, C18, 110A˚, 5µm, 150 * 3 mm
Buffer	:	5 mM Ammonium Acetate (pH 4.0)
Mobile phase	:	Pump A: Buffer, Pump B: Acetonitrile In the ratio. 25:75
Flow rate	:	0.500 mL/minute
Injection volume	:	10.000 µL
Auto sampler temperature	:	10°C
Column Temperature	:	35°C
Rinsing solution	:	Acetonitrile
Run time	:	3.00 minutes
Retention time	:	Ezetimibe at around 1.96 minutes Ezetimibe-D4 at around 1.94 minutes.

RESULT AND DISCUSSION

SYSTEM SUITABILITY TEST

This test was performed in order to check the suitability of the system with optimized final condition and to maintain performance of the system reproducibility in changing environment. The percent co-efficient of variation for peak area ratio of analyte to internal standard and for retention time were under the acceptance criteria i.e. % C.V was less than 5 % for LC-MS/MS based procedures. Retention time for drug and IS was around 1.96 min and 1.94 min respectively.

Table No. 6 System Suitability Test

S. No.	Analyte Area	IS area	Area ratio	RT of analyte	RT of IS
1	859870	80106	10.734	1.93	1.92
2	866907	80724	10.739	1.93	1.92
3	887991	83353	10.653	1.93	1.92
4	885149	82373	10.746	1.93	1.92
5	875909	82372	10.634	1.93	1.92
6	867605	81671	10.623	1.93	1.92
Mean	873905.2	81766.5	10.6882	1.930	1.920
S.D±	11086.05	1192.03	0.05735	0.0000	0.0000
%CV	1.27	1.46	0.54	0.00	0.00

SELECTIVITY

The selectivity is generally defined as the lack of interfering peaks at the retention time of the assayed drug and the internal standard in the chromatograms. Six lots of human blank plasma (four normal, one lipemic and one hemolysed)

containing K2EDTA as an anticoagulant was evaluated for their selectivity and no interference was observed at the retention time of Ezetimibe and Ezetimibe D4 (Internal Standard) in any of the human blank plasma lots.

Acceptance Criteria

1. % Responses of interfering peaks at the RT of analyte should be < 20% response of the analyte in LLOQ.

2. % Responses of interfering peaks at the RT of IS should be < 5% response of the IS in LLOQ.

TABLE No: 7 Selectivity

Batch No./ Lot No.	Analyte Response		% Interference at RT of Analyte	IS Response		% Interference at RT of IS
	Blank	LLOQ		Blank	LLOQ	
RCT/ML-001	81	4731	1.71	0	83892	0.00
RCT/ML-002	80	4709	1.70	0	88530	0.00
RCT/ML-003	213	4698	4.53	0	77959	0.00
RCT/ML-004	0	3925	0.00	0	75396	0.00
LP021	0	5016	0.00	0	92684	0.00
HP030	232	5033	4.61	231	89807	0.26

CARRY OVER

The experiment was performed to check the carryover of the assay method and carryover of the instrument. The carry over experiment was performed by injecting extracted samples in the following sequence: Blank, LLOQ samples in duplicate, ULOQ samples in duplicate and Blank (Duplicate injection from same vial). Both the LLOQ samples were used for the evaluation of carry over effect.

From results it can be concluded that, there was no carry-over or contamination observed at the retention time of Ezetimibe and Ezetimibe D4 (Internal Standard).

Acceptance Criteria

1. Response of interfering peaks at the retention time of analyte in the blank sample injected after ULOQ sample should be within 20 % of the mean response of the LLOQ sample.

2. Response of interfering peaks at the retention time of internal standard in the blank sample injected after ULOQ sample should be within 5 % of the mean response of the LLOQ sample.

Table No. 8 Carryover

Sample ID	Analyte Response	Mean Response of Analyte at LLOQ	IS Response	Mean Response of IS at LLOQ	% Carryover	
					Analyte	IS
BLANK-01	60	4122.5	337	76075.5	N/AP	N/AP
LLOQ-01	4075		72373			
LLOQ-02	4170		79778			
ULOQ-01	2234166		83799			
ULOQ-02	2184166		82102			
BLANK-01	0		354		0.00	0.47
BLANK-01	52		306		1.26	0.40

MATRIX EFFECT

Matrix factor is the quantitative measure of the matrix effect due to suppression or enhancement of ionization in mass spectrometric detector. Matrix effect was evaluated through matrix factor, which is calculated by comparing area response in presence of matrix ions with mean area response in absence of matrix ions.

1. Area response in absence of matrix ion:

The extracted aqueous quality control samples were prepared at two distinct levels of LQC and HQC, by spiking the appropriate volume of respective spiking solutions into water and processed as per extraction procedure. Six replicate of low

and high level of quality control samples were are prepared. The mean area response of these six injections was considered as area response in absence of matrix ions.

2. Area response in presence of matrix ion:

Six lots of K2EDTA blank plasma samples (four normal, one hemolysed and one lipemic) were taken. Two replicates from six lots of K2EDTA blank plasma samples along with six replicates of low and high level of quality control samples were prepared in water and processed as per extraction procedure.

Thereafter, quality control samples were prepared at two distinct levels of LQC & HQC by reconstituting the above processed blank samples with respective processed aqueous quality control samples.

Single injection was given from each lot and obtained area response at low and high level of quality control samples was considered as area response in presence of matrix ions. The matrix factor was derived using below mentioned equation for analyte and internal standard.

Matrix factor =	Peak area in presence of matrix ions
	Mean peak area in absence of matrix ions

The IS normalized matrix factor was derived using below mentioned equation.

IS Normalized MF =	Matrix factor of analyte
	Matrix factor of internal standard

Acceptance Criteria:

The % CV for IS normalized matrix factors in six lots should be within 15% at LQC and HQC level.

Table No. 9 Matrix Effect

PEAK AREA IN ABSENCE OF MATRIX IONS									
S. No	**LQC**					**HQC**			
	Analyte		**Internal Standard**			**Analyte**		**Internal Standard**	
1	9390		97476			1635886		103609	
2	7871		79079			1435663		85482	
3	10256		102990			2000963		122192	
4	10194		103758			1975642		119796	
5	8280		85501			1622051		98392	
6	7204		80496			1965879		124131	
Mean	**8865.8**		**91550.0**			**1772680.7**		**108933.7**	
SD	**1270.11**		**11219.03**			**239004.06**		**15583.27**	
% CV	**14.33**		**12.25**			**13.48**		**14.31**	
PEAK AREA IN PRESENCE OF MATRIX IONS									
Blank Matrix Batch / Lots No.	**LQC**					**HQC**			
	Peak area		**Matrix factor**		**IS Normalized MF**	**Peak area**		**Matrix factor**		**IS Normalized MF**
	Analyte	**Internal Standard**	**Analyte**	**Internal Standard**		**Analyte**	**Internal Standard**	**Analyte**	**Internal Standard**	
RCT/ML-001	6596	66851	0.74	0.73	1.02	1390965	88620	0.78	0.81	0.96
RCT/ML-002	5586	62724	0.63	0.69	0.92	1019546	68281	0.58	0.63	0.92
RCT/ML-003	5830	55485	0.66	0.61	1.09	1033654	67095	0.58	0.62	0.95
RCT/ML-004	8618	83843	0.97	0.92	1.06	893356	55829	0.50	0.51	0.98
LP021	6681	67911	0.75	0.74	1.02	914633	58185	0.52	0.53	0.97
HP030	6996	63681	0.79	0.70	1.13	1236917	71767	0.70	0.66	1.06
Mean					**1.039**	**Mean**				**0.973**
SD					**0.0734**	**SD**				**0.0478**
% CV					**7.06**	**% CV**				**4.91**

PRECISION AND ACCURACY

Precision and accuracy of analyte was determined for LLOQ QC, LQC, MQC1, MQC and HQC samples in the biological matrix, based on the expected range.

Precision

Precision of the assay was measured by the percent coefficient of variance over the concentration range of lower limit of quantification, low, medium and high quality control samples of analyte during the course of the validation.

Within-batch precision (Intra-day precision)	1.37 % to 4.85 %.
Between-batch precision (Inter-day precision)	1.79 % to 6.84 %.

Accuracy

Accuracy of the assay was defined as the absolute value of the ratio of the mean back-calculated values of the quality control samples to their respective nominal values, expressed in percentage.

Within-batch accuracy (Intra-day accuracy)	84.34 % to 99.96 %.
Between-batch accuracy (Inter-day accuracy)	93.27 % to 99.14 %.

Acceptance Criteria for Quality Control Samples

1. Accuracy of at least 50 % of QC samples at each level and at least 67 % of total QC samples should be within or equal to ± 15 % of their respective nominal concentration except LLOQQC, for which it should be within or equal to ± 20 % of the nominal concentration.

2. The precision (% CV) determined at each QC level should be within or equal to 15 %, except LLOQQC, for which it should be within or equal to 20 %.

3. Intraday (within run) and inter day (between run) accuracy should be within or equal to ± 20 % for LLOQQC, and for other quality control samples accuracy should be within or equal to ± 15 %.

4. Intraday (within run) and inter day (between run) precision should be within or equal to 20 % for LLOQ QC, and for other quality control samples precision should be within or equal to 15 %.

Table No. 10: Within and Between batch Precision and Accuracy

Quality Control ID	**LLOQQC**	**LQC**	**MQC1**	**MQC**	**HQC**
Nominal Concentration ng/mL	**0.513**	**1.231**	**41.025**	**112.820**	**205.127**
Acceptance Range	**0.410**	**1.046**	**34.871**	**95.897**	**174.358**
	0.616	**1.416**	**47.179**	**129.743**	**235.896**
Back Calculated Concentration (ng/mL)					
P and A-01					
1	0.466	1.193	39.813	111.198	201.613
2	0.410	1.154	40.724	109.611	194.748
3	0.445	1.177	42.421	114.431	202.518
4	0.413	1.177	41.062	112.810	204.386
5	0.435	1.153	41.730	117.418	199.569
6	0.427	1.183	39.653	111.163	193.098
Mean	**0.4327**	**1.1728**	**40.9005**	**112.7718**	**199.3220**
±SD	**0.02098**	**0.01608**	**1.07667**	**2.80704**	**4.49061**
Precision (%CV)	**4.85**	**1.37**	**2.63**	**2.49**	**2.25**
Accuracy (%)	**84.34**	**95.27**	**99.70**	**99.96**	**97.17**
P and A-02					
1	0.495	1.274	40.136	115.159	203.791
2	0.484	1.261	41.325	109.041	195.958
3	0.482	1.183	39.402	108.303	201.843
4	0.478	1.174	40.563	109.631	202.259
5	0.505	1.177	38.491	114.282	206.139
6	0.487	1.251	40.429	116.344	206.901

Quality Control ID	**LLOQQC**	**LQC**	**MQC1**	**MQC**	**HQC**
Nominal Concentration ng/mL	**0.513**	**1.231**	**41.025**	**112.820**	**205.127**
Acceptance Range	**0.410**	**1.046**	**34.871**	**95.897**	**174.358**
	0.616	**1.416**	**47.179**	**129.743**	**235.896**
Back Calculated Concentration (ng/mL)					
Mean	**0.4885**	**1.2200**	**40.0577**	**112.1267**	**202.8152**
±SD	**0.00989**	**0.04667**	**0.98893**	**3.52126**	**3.92350**
Precision (%CV)	**2.03**	**3.83**	**2.47**	**3.14**	**1.93**
Accuracy (%)	**95.22**	**99.11**	**97.64**	**99.39**	**98.87**
P and A-03					
1	0.491	1.191	40.917	108.927	199.146
2	0.474	1.236	40.501	111.092	199.622
3	0.478	1.252	39.555	109.423	202.257
4	0.484	1.303	39.050	112.421	198.171
5	0.468	1.203	40.491	109.795	195.274
6	0.481	1.224	41.420	106.100	200.287
Mean	**0.4793**	**1.2348**	**40.3223**	**109.6263**	**199.1262**
±SD	**0.00799**	**0.03999**	**0.87481**	**2.14253**	**2.33013**
Precision (%CV)	**1.67**	**3.24**	**2.17**	**1.95**	**1.17**
Accuracy (%)	**93.44**	**100.31**	**98.29**	**97.17**	**97.07**
P and A-04					
1	0.512	1.278	39.146	109.081	197.489
2	0.506	1.244	41.423	111.654	203.521
3	0.527	1.250	40.271	110.580	196.776
4	0.534	1.239	39.819	106.853	202.555
5	0.494	1.250	40.513	110.605	198.393
6	0.508	1.264	39.955	110.780	200.465
Mean	**0.5135**	**1.2542**	**40.1878**	**109.9255**	**199.8665**
±SD	**0.01464**	**0.01437**	**0.76341**	**1.71822**	**2.76827**
Precision (%CV)	**2.85**	**1.15**	**1.90**	**1.56**	**1.39**
Accuracy (%)	**100.10**	**101.88**	**97.96**	**97.43**	**97.44**
Global Statistics					
Quality Control ID	**LLOQQC**	**LQC**	**MQC1**	**MQC**	**HQC**
Mean	0.4785	1.2205	40.3671	111.1126	200.2825
±SD	0.03273	0.04318	0.93047	2.82462	3.58950
Precision (%CV)	6.84	3.54	2.31	2.54	1.79
Accuracy (%)	93.27	99.14	98.40	98.49	97.64

Note: Attachment 02 includes representative chromatograms of P and A Batch.

RECOVERY

The recovery was determined by comparing the detector response of analyte at three distinct levels of extracted low medium and high quality control samples with detector response obtained from the recovery samples. Recovery samples were prepared by processing aqueous low, medium and high quality control samples.

Based on the results, it was concluded that recovery is precise for the analyte at all three QC levels.

For Analyte

The mean % Absolute Recovery for analyte at low, medium and high quality control samples was 84.76 %, 84.37 % and 86.69 % respectively. The % CV for average of mean % Absolute Recoveries was 1.39 %.

Acceptance Criteria

The % CV of % absolute recovery and overall % absolute recovery for analyte should be within 15% for each quality control.

Table No.11: Recovery of Ezetimibe

	Extracted LQC	**Un extracted LQC**	**Extracted MQC**	**Un extracted MQC**	**Extracted HQC**	**Un extracted HQC**
	6349	8059	611480	727828	1251206	1499562
	6441	7019	610956	756142	1242757	1315405
	6407	8121	598332	693234	1322207	1384079
	6692	8099	646866	716286	1295590	1444124
	6932	7861	544556	749427	1204762	1378071
	7116	7961	653388	701992	1029404	1462504
	Mean	**7853.3**	**Mean**	**724151.5**	**Mean**	**1413957.5**
	SD	**420.01**	**SD**	**25237.12**	**SD**	**67064.26**
	% CV	**5.35**	**% CV**	**3.49**	**% CV**	**4.74**
	% Absolute Recovery					
	LQC		**MQC**		**HQC**	
	80.84		84.44		88.49	
	82.02		84.37		87.89	
	81.58		82.63		93.51	
	85.21		89.33		91.63	
	88.27		75.20		85.20	
	90.61		90.23		72.80	
Mean	**84.756**		**84.365**		**86.588**	
SD	**3.9925**		**5.4046**		**7.3565**	
%CV	**4.71**		**6.41**		**8.50**	
Overall % Absolute Recovery						
Global Mean	**85.2364**					
Global SD	**1.18698**					
Global %CV	**1.39**					

For Internal standard

The mean % Absolute Recovery for internal standard at low, medium and high quality control samples was 86.56 %, 84.74 % and 86.92 % respectively. The % CV for average of mean of % Absolute Recoveries was 1.36 %.

Acceptance Criteria

The % CV of % absolute recovery and overall % absolute recovery for internal standard should be within 15% for each quality control.

Table No. 12: Recovery of Ezetimibe D4

	Extracted LQC	Un extracted LQC	Extracted MQC	Un extracted MQC	Extracted HQC	Un extracted HQC
	58863	72298	63491	75404	71628	87168
	61618	65004	62071	77731	74549	76123
	57657	73813	62414	69272	76678	80009
	60507	70387	66938	74729	76521	81963
	64532	72468	55707	78038	67859	79471
	66488	73097	67811	71381	58424	84965
	Mean	**71177.8**	**Mean**	**74425.8**	**Mean**	**81616.5**
	SD	**3233.83**	**SD**	**3488.66**	**SD**	**3987.92**
	% CV	**4.54**	**% CV**	**4.69**	**% CV**	**4.89**
	% Absolute Recovery					
	LQC		**MQC**		**HQC**	
	82.70		85.31		87.76	
	86.57		83.40		91.34	
	81.00		83.86		93.95	
	85.01		89.94		93.76	
	90.66		74.85		83.14	
	93.41		91.11		71.58	
Mean	**86.559**		**84.745**		**86.923**	
SD	**4.7320**		**5.8028**		**8.5541**	
%CV	**5.47**		**6.85**		**9.84**	
Overall % Absolute Recovery						
Global Mean	**86.0755**					
Global SD	**1.16666**					
Global %CV	**1.36**					

DILUTION INTEGRITY

Dilution integrity for analyte was evaluated by preparing sample around 3 to 4 times higher than the concentration of ULOQ. A sample of analyte having concentration of 820.508 ng/mL was prepared in human plasma and this was diluted with human blank plasma to 1/4 and 1/10 of the original concentration and analyzed using a set of calibration curve standards. Precision and Accuracy of the dilution integrity samples for 1/4 and 1/10 dilutions were found within the acceptance criteria.

Precision	for 1/4 dilution integrity samples	4.16 %
	for 1/10 dilution integrity samples	3.18 %
Accuracy	for 1/4 dilution integrity samples	96.21 %
	for 1/10 dilution integrity samples	100.85 %

Table No. 13: Dilution Integrity

Nominal Concentration (ng/mL)	**820.508**	**820.508**
Acceptance Range	697.432	697.432
	943.584	943.584
Dilution Factor	**Quality Control (1/4)**	**Quality Control (1/10)**
Back Calculated Concentration (ng/mL)	757.160	787.351
	786.564	804.611
	799.820	850.187
	798.195	835.155
	842.209	833.341
	752.507	854.461
Mean	**789.4092**	**827.5177**
SD	**32.81241**	**26.34448**
% CV	**4.16**	**3.18**
%Accuracy	**96.21**	**100.85**

Ruggedness

To evaluate the ruggedness of method, one P and A batch was processed by different analyst and analyzed on different column (same type) with fresh reagents and solutions. The Ruggedness precision and accuracy batch was found to be within acceptance criteria. Based on the results, it was concluded that the method is rugged.

within batch precision of quality control samples	1.21 % to 5.09 %,
within batch accuracy of quality control samples	102.34 % to 107.08 %

Table No. 14 : Ruggedness

Quality Control ID	LLOQQC	LQC	MQC1	MQC	HQC
Nominal Concentration ng/mL	0.513	1.231	41.025	112.820	205.127
Acceptance Range	0.410	1.046	34.871	95.897	174.358
	0.616	1.416	47.179	129.743	235.896
Back Calculated Concentration (ng/mL)	0.571	1.352	42.484	116.040	213.061
	0.576	1.296	41.214	115.874	209.857
	0.510	1.316	42.509	116.556	207.939
	0.520	1.305	41.358	119.587	209.081
	0.543	1.292	43.278	115.928	227.448
	0.566	1.348	41.055	116.588	213.874
Mean	**0.5477**	**1.3182**	**41.9830**	**116.7622**	**213.5433**
±SD	**0.02789**	**0.02603**	**0.89976**	**1.41836**	**7.19155**
Precision (%CV)	**5.09**	**1.98**	**2.14**	**1.21**	**3.37**
Accuracy (%)	**106.76**	**107.08**	**102.34**	**103.49**	**104.10**

Reinjection Reproducibility

To estimate the reinjection reproducibility, samples of Precision and Accuracy-03 was re-injected and it was found within acceptance criteria.

Based on the results, the reinjection reproducibility was proved.

within batch precision of quality control samples	2.17 % to 5.22%,
within batch accuracy of quality control samples	94.48 % to 100.04 %

Table No. 15: Reinjection Reproducibility

Nominal Concentration	**LLOQQC**	**LQC**	**MQC1**	**MQC**	**HQC**
	0.513	1.231	41.025	112.820	205.127
Acceptance Range	0.410	1.046	34.871	95.897	174.358
	0.616	1.416	47.179	129.743	235.896
Back Calculated Concentration (ng/mL)	0.492	1.200	38.606	109.058	192.385
	0.502	1.247	40.936	110.722	191.783
	0.456	1.267	40.129	110.625	197.448
	0.478	1.221	39.181	106.252	202.220
	0.521	1.168	40.552	111.607	198.767
	0.459	1.286	39.991	114.758	201.564
Mean	**0.4847**	**1.2315**	**39.8992**	**110.5037**	**197.3612**
±SD	**0.02530**	**0.04383**	**0.86608**	**2.81232**	**4.45273**
Precision (%CV)	**5.22**	**3.56**	**2.17**	**2.55**	**2.26**
Accuracy (%)	**94.48**	**100.04**	**97.26**	**97.95**	**96.21**

Hemolysis Effect

Hemolysis effect has carried out to assess the impact on the accuracy and precision of the method, because of hemolysed matrix. Six replicates of LQC and HQC was prepared in one of the hemolysed matrix lot and analyzed against bulk spiked calibration standard.

Based on the results, it was concluded that there is no hemolytic effect.

within batch precision	LQC: 3.44 % and HQC: 1.68 %,
within batch accuracy	LQC: 98.06 % and HQC: 95.76 %

Table No. 16: Hemolysis effect

Quality Control Sample	HE_LQC	HE_HQC
Nominal Concentration ng/mL	1.231	205.127
Acceptance Range	1.046	174.358
	1.416	235.896
Back Calculated Concentration (ng/mL)	1.140	194.477
	1.242	201.220
	1.239	193.498
	1.179	197.122
	1.203	193.097
	1.240	199.161
Mean	**1.2072**	**196.4292**
SD	**0.04154**	**3.29852**
% CV	**3.44**	**1.68**
% Accuracy	**98.06**	**95.76**
N	**6**	**6**

Reagent stability

The stability of the reagents which were used during the method validation was evaluated. The reagents were kept on bench top at room temperature on 16Oct2017.After 12 days, six sets of LQC and HQC samples were processed using old reagents (prepared on 16Oct2017) and simultaneously calibration curve standards and another six sets of LQC and HQC samples were processed using freshly prepared reagents (prepared on 28Oct2017) and analyzed.

Based on the results, it was concluded that the below mentioned reagents were stable up to 12 days at room temperature.

Note: Enzyme Solution and 1.00% L-Ascorbic acid were prepared on the day of usage.

Reagent used for processing:

- 5mM Ammonium Acetate Buffer
- 0.5 M Sodium Acetate Buffer
- 1.00% L-ascorbic Acid
- β-Glucuronidase Enzyme Solution
- 1M Sodium Hydroxide Buffer
- Mobile Phase (ACN: Buffer :: 75:25%v/v)

Within Batch Precision	LQC: 2.86% and HQC: 2.65%
% Change	LQC: 0.15% and HQC: 2.91%

Acceptance Criteria

The % change should not be more than ± 15% while comparing the mean of back calculated concentration of stability samples against the nominal concentration.

Table No. 17: Reagent stability for Ezetimibe (After 12 days)

Quality Control ID	Fresh LQC	Fresh HQC	Stability LQC	Stability HQC
Nominal Concentration ng/mL	1.231	205.127	1.231	205.127
Acceptance Range	1.046	174.358	1.046	174.358
	1.416	235.896	1.416	235.896
Back Calculated Concentration (ng/mL)	1.243	206.576	1.253	217.310
	1.242	203.607	1.181	208.444
	1.260	205.562	1.198	217.558
	1.194	205.672	1.267	205.187
	1.236	199.558	1.259	205.492
	1.308	202.963	1.239	212.620
Mean	**1.2472**	**203.9897**	**1.2328**	**211.1018**
SD	**0.03704**	**2.56249**	**0.03520**	**5.58655**
% Accuracy	**101.31**	**99.45**	**100.15**	**102.91**
% CV	**2.97**	**1.26**	**2.86**	**2.65**
% Change			**0.15**	**2.91**

STABILITIES:

1. Short Term Stock Solution/Dilution Stability at Room Temperature

a. Short Term Stock Solution Stability for Ezetimibe and Ezetimibe D4 (Internal Standard)

Diluent- Methanol: Water (80:20)

Analyte

The stock solutions of Ezetimibe was prepared and kept on the bench at room temperature. After 29 Hrs stock solution of Ezetimibe was compared against fresh stock solution.

The % difference for stock solution of Ezetimibe was 0.16 %and 1.05 % at LLOQ and ULOQ Level respectively.

Internal standard

The stock solutions of Ezetimibe D4 was prepared and kept on the bench at room temperature. After 29 Hrs stock solution of Ezetimibe D4 was compared against fresh stock solution.

The % difference for stock solutions of EzetimibeD4 at working solution concentration level was 1.94 %.

Acceptance Criteria

The % difference for stability solution should be within ±10 % of response compared with the response of freshly prepared solutions.

Table No. 18: Short Term Stock Solution Stability of Ezetimibe and EzetimibeD4

Concentration (ng/mL)	Analyte				Internal Standard	
	LLOQ		ULOQ		Stock Dilution at working concentration	
Fresh Stock	0.504		250.915		12.766	
Stability Stock	0.504		250.948		12.650	
Correction factor	1.000		1.000		1.009	
	Fresh Stock	Stability Stock	Fresh Stock	Stability Stock	Fresh Stock	Stability Stock
Area Response	5911	5871	2446718	2442159	111239	111659
	6121	6300	2402585	2398532	107223	107907
	6176	6124	2296983	2328864	103635	105048
	6097	5769	2311450	2261491	104433	102343
	5620	5631	2347626	2376376	107153	107727
	5549	5837	2343762	2492601	105709	111203
Mean	**5912.3**	**5922.0**	**2358187.3**	**2383337.2**	**106565.3**	**107647.8**
SD	**270.05**	**245.60**	**56672.41**	**81843.45**	**2701.41**	**3569.38**
%CV	**4.57**	**4.15**	**2.40**	**3.43**	**2.53**	**3.32**
% Difference	**0.16**		**1.05**		**1.94**	

b. Short Term Stock Solution Stability of Ezetimibe Phenoxy Glucuronide

Diluent- Methanol: Water (80:20)

Metabolite

The stock solutions of Ezetimibe Phenoxy Glucuronide was prepared and kept on the bench at room temperature. After 49 Hrs stock solution of Ezetimibe Phenoxy Glucuronide was compared against fresh stock solution.

For Ezetimibe Phenoxy Glucuronide at LLOQ QC and HQC levels (equivalent to Ezetimibe concentration),one sample per each solution was processed (Respective solutions were spiked in water and extracted as per extraction procedure) and six replicate injections were given from each vial for fresh and stability solutions.

The % difference for stock solution of Ezetimibe at LLOQQC and HQC level were -2.82 % and -3.83 % respectively.

Acceptance Criteria

The % difference for stability solution should be within ±10 % of response compared with the response of freshly prepared solutions.

Table No. 19: Short Term Stock Solution Stability of Ezetimibe Phenoxy Glucuronide

Concentration (ng/mL)	Metabolite			
	LLOQQC		HQC	
Fresh Stock	0.503		201.395	
Stability Stock	0.505		202.679	
Correction factor	0.996		0.994	
	Fresh Stock	Stability Stock	Fresh Stock	Stability Stock
Area Response	4410	4559	1522035	1475600
	4626	4254	1505906	1469517
	4280	4449	1533091	1492721
	4396	4101	1537246	1493528
	4436	4354	1566038	1518223
	4636	4414	1589005	1506287
Mean	**4464.0**	**4355.2**	**1542220.2**	**1492646.0**
SD	**140.01**	**160.41**	**30299.68**	**18256.83**
%CV	**3.14**	**3.68**	**1.96**	**1.22**
% Difference	**-2.82**		**-3.83**	

c. Short Term Stock Dilution Stability for Ezetimibe and Ezetimibe D4 (Internal Standard)

Diluent- Methanol: Water (80:20)

Analyte

The working solution of Ezetimibe at ULOQ and LLOQ concentration was prepared and kept on the bench at room temperature. After 26 Hrs the working solution at ULOQ and LLOQ concentration was compared against freshly spiked working solution.

The % difference for the working solution of Ezetimibe at LLOQ and ULOQ concentration was -0.33 % and 1.16% respectively.

Internal Standard

The working solution of Ezetimibe D4 was prepared and kept on the bench at room temperature. After 26 Hrs the working solution was compared against fresh working solution. The % difference for the working solution of internal standard was 2.54 %. **Acceptance Criteria**

The % difference for stability solution should be within ±10 % of response compared with the response of freshly prepared solutions.

Table No. 20: Short Term Stock Dilution Stability of Ezetimibe and Ezetimibe D4

Concentration (ng/mL)	**Analyte**				**Internal Standard**	
	LLOQ		**ULOQ**		**Stock Dilution at working concentration**	
Fresh Stock	0.504		250.915		12.766	
Stability Stock	0.504		250.948		12.650	
Correction factor	1.000		1.000		1.009	
	Fresh Stock	**Stability Stock**	**Fresh Stock**	**Stability Stock**	**Fresh Stock**	**Stability Stock**
Area Response	5911	6081	2446718	2390907	111239	109168
	6121	6286	2402585	2326714	107223	107207
	6176	5610	2296983	2360634	103635	108704
	6097	5828	2311450	2298235	104433	103207
	5620	5909	2347626	2480492	107153	110470
	5549	5642	2343762	2457644	105709	110895
Mean	**5912.3**	**5892.7**	**2358187.3**	**2385771.0**	**106565.**	**108275.2**
SD	**270.05**	**259.76**	**56672.41**	**72036.55**	**2701.41**	**2810.31**
%CV	**4.57**	**4.41**	**2.40**	**3.02**	**2.53**	**2.60**
% Difference	**-0.33**		**1.16**		**2.54**	

d. Short Term Stock Dilution Stability of Ezetimibe Phenoxy Glucuronide

Diluent- Methanol: Water (80:20)

Metabolite

The working solution of Ezetimibe Phenoxy Glucuronide at LLOQQC and HQC concentration was prepared and kept on the bench at room temperature. After 47 Hrs the working solution at LLOQQC and HQC concentration was compared against freshly spiked working solution.

For Ezetimibe Phenoxy Glucuronide at LLOQ QC and HQC levels (equivalent to Ezetimibe concentration),one sample per each solution was processed (Respective solutions were spiked in water and extracted as per extraction procedure)and six replicate injections were given from each vial for fresh and stability solutions.

The % difference for stock solution of Ezetimibe at LLOQQC and HQC level were -2.27 % and 1.22 % respectively.

Acceptance Criteria

The % difference for stability solution should be within ±10 % of response compared with the response of freshly prepared solutions.

Table No. 21: Short Term Stock Dilution Stability of Ezetimibe Phenoxy Glucuronide

Concentration (ng/mL)	Metabolite			
	LLOQQC		HQC	
Fresh Stock	0.505		201.915	
Stability Stock	0.509		199.763	
Correction factor	0.992		1.011	
	Fresh Stock	**Stability Stock**	**Fresh Stock**	**Stability Stock**
Area Response	4224	4312	1630875	1605139
	4208	4218	1618657	1616316
	4241	4294	1604685	1644808
	4378	4427	1625562	1630306
	4602	4117	1613879	1623985
	4377	4272	1639016	1625392
Mean	**4338.3**	**4273.3**	**1622112.3**	**1624324.3**
SD	**149.75**	**102.99**	**12311.44**	**13332.86**
%CV	**3.45**	**2.41**	**0.76**	**0.82**
% Difference	**-2.27**		**1.22**	

2. Long Term Stock Solution / Dilution Stability At Refrigerator

a. Long Term Stock Solution Stability for Ezetimibe and Ezetimibe D4

Diluent: Methanol

Analyte

The stock solutions of Ezetimibe was prepared and stored at refrigerator temperature (2-8ºC). After 18 days the stock solutions of Ezetimibe was compared against fresh stock solutions of Ezetimibe.

The % difference for stock solution of Ezetimibe at LLOQ and ULOQ level were -2.20 % and 0.94 % respectively.

Internal Standard

The stock solutions of Ezetimibe D4 was prepared and stored at refrigerator temperature (2-8ºC). After 18 days the stock solutions of Ezetimibe D4 was compared against fresh stock solutions of Ezetimibe D4.

The % difference for stock solution of Ezetimibe D4 was 1.43 % at working solution concentration level.

Acceptance Criteria

The % difference for stability solution should be within ± 10 % of response compared with the response of freshly prepared solutions.

Table No. 22: Long Term Stock Solution Stability for Ezetimibe and Ezetimibe D4

Concentration (ng/mL)	Analyte				Internal Standard	
	LLOQ		ULOQ		Stock Dilution at working	
Fresh Stock	0.504		250.915		12.766	
Stability Stock	0.503		251.703		12.788	
Correction factor	1.002		0.997		0.998	
	Fresh Stock	**Stability Stock**	**Fresh Stock**	**Stability Stock**	**Fresh Stock**	**Stability Stock**
Area Response	5911	6282	2446718	2392350	111239	107037
	6121	5761	2402585	2320336	107223	104695
	6176	5811	2296983	2311075	103635	105312
	6097	5771	2311450	2474739	104433	112182
	5620	5658	2347626	2417566	107153	110351
	5549	5341	2343762	2410384	105709	110097
Mean	**5912.3**	**5770.7**	**2358187.3**	**2387741.7**	**106565.3**	**108279.0**
SD	**270.05**	**303.48**	**56672.41**	**62310.90**	**2701.41**	**3033.89**
%CV	**4.57**	**5.26**	**2.40**	**2.61**	**2.53**	**2.80**
% Difference	**-2.20**		**0.94**		**1.43**	

b. Long Term Stock Solution Stability of Ezetimibe Phenoxy Glucuronide

Diluent- Methanol: Water (80:20)

Metabolite

The stock solutions of Ezetimibe Phenoxy Glucuronide was prepared and stored at refrigerator temperature (2-8ºC). After 20 days the stock solutions of ezetimibe phenoxy glucuronide was compared against fresh stock solutions of ezetimibe phenoxy Glucuronide.

For ezetimibe phenoxy glucuronide at LLOQ QC and HQC levels (equivalent to Ezetimibe concentration), one sample per each solution was processed (Respective solutions were spiked in water and extracted as per extraction procedure) and six replicate injections were given from each vial for fresh and stability solutions.

The % difference for stock solution of Ezetimibe at LLOQQC and HQC level were 3.25% and -7.01% respectively.

Acceptance Criteria

The % difference for stability solution should be within ±10 % of response compared with the response of freshly prepared solutions.

Table No. 23: Long Term Stock Solution Stability of Ezetimibe Phenoxy Glucuronide

Concentration (ng/mL)	Metabolite			
	LLOQQC		HQC	
Fresh Stock	0.503		201.395	
Stability Stock	0.504		201.485	
Correction factor	0.998		1.000	
	Fresh Stock	Stability Stock	Fresh Stock	Stability Stock
Area Response	4410	4550	1522035	1380058
	4626	4637	1505906	1443269
	4280	4618	1533091	1431025
	4396	4401	1537246	1445073
	4436	4820	1566038	1475458
	4636	4683	1589005	1433201
Mean	**4464.0**	**4618.2**	**1542220.2**	**1434680.7**
SD	**140.01**	**139.36**	**30299.68**	**31124.72**
%CV	**3.14**	**3.02**	**1.96**	**2.17**
% Difference	**3.25**		**-7.01**	

b. Long term Stock Dilution stability for Ezetimibe and Ezetimibe D4

Diluent- Methanol: Water (80:20)

Analyte

The working solutions of Ezetimibe at ULOQ and LLOQ concentration was prepared and stored in refrigerator temperature (2-8ºC). After 18 days the working solution of Ezetimibe at ULOQ and LLOQ concentration was compared against freshly spiked working solution.

The % difference for stock solution of Ezetimibe at LLOQ and ULOQ level were -0.85 % and 2.37 % respectively.

Internal Standard

Working solution of Ezetimibe D4 at working solution concentration level was prepared and stored at refrigerator temperature (2-8ºC). After 18 days the working solution of Ezetimibe D4 was compared against fresh working solution.

The % difference for the working solution of internal standard was 3.08 %.

Table No. 24: Long term Stock Dilution stability for Ezetimibe and Ezetimibe D4

Concentration (ng/mL)	Analyte				Internal Standard	
	LLOQ		ULOQ		Stock Dilution at working concentration	
Fresh Stock	0.504		250.915		12.766	
Stability Stock	0.503		251.703		12.788	
Correction factor	1.002		0.997		0.998	
	Fresh Stock	**Stability Stock**	**Fresh Stock**	**Stability Stock**	**Fresh Stock**	**Stability Stock**
Area Response	5911	6284	2446718	2386247	111239	111115
	6121	5942	2402585	2376302	107223	107225
	6176	5784	2296983	2399365	103635	107228
	6097	5814	2311450	2489216	104433	112496
	5620	5809	2347626	2436465	107153	110360
	5549	5469	2343762	2441644	105709	111808
Mean	**5912.3**	**5850.3**	**2358187.3**	**2421539.8**	**106565.3**	**110038.7**
SD	**270.05**	**264.32**	**56672.41**	**42404.65**	**2701.41**	**2291.17**
%CV	**4.57**	**4.52**	**2.40**	**1.75**	**2.53**	**2.08**
% Difference	**-0.85**		**2.37**		**3.08**	

c. Long Term Stock Dilution Stability of Ezetimibe Phenoxy Glucuronide

Diluent- Methanol: Water (80:20)

Metabolite

The working solution of ezetimibe phenoxy glucuronide at LLOQQC and HQC concentration was prepared and stored at refrigerator temperature (2-8ºC).

After 20 days the working solution at LLOQQC and HQC concentration was compared against freshly spiked working solution.

Ezetimibe phenoxy glucuronide at LLOQ QC and HQC levels, one sample per each solution are processed (Respective solutions are spiked in water and extracted as per extraction procedure.) and six replicate injections were given from each vial for fresh and stability solutions.

The % difference for stock solution of Ezetimibe Phenoxy Glucuronide at LLOQQC and HQC level were -1.57 % and 3.77 % respectively.

Acceptance Criteria

The % difference for stability solution should be within ±10 % of response compared with the response of freshly prepared solutions.

Table No. 25 : Long term Stock Dilution stability for Ezetimibe Phenoxy Glucuronide

Concentration (ng/mL)	**Metabolite**			
	LLOQQC		**HQC**	
Fresh Stock	0.505		201.915	
Stability Stock	0.516		198.300	
Correction factor	0.979		1.018	
	Fresh Stock	**Stability Stock**	**Fresh Stock**	**Stability Stock**
Area Response	4224	4146	1630875	1626546
	4208	4275	1618657	1630614
	4241	4336	1604685	1640935
	4378	4540	1625562	1665148
	4602	4383	1613879	1663618
	4377	4499	1639016	1692177
Mean	**4338.3**	**4363.2**	**1622112.3**	**1653173.0**
SD	**149.75**	**145.46**	**12311.44**	**25065.85**
%CV	**3.45**	**3.33**	**0.76**	**1.52**
% Difference	**-1.57**		**3.77**	

3. Auto Sampler Stability

The Auto sampler stability of analyte for low and high quality control samples were evaluated by analyzing them using a set of freshly spiked and prepared calibration curve standards. The stability samples were stored in the auto-sampler (10°C temperature).

Based on the results, it was concluded that the analyte were stable at auto sampler temperature (10°C) for 124 Hrs 38 Min.

Acceptance Criteria

The % change should not be more than ±15% while comparing the mean of back calculated concentration of stability samples against the nominal concentration.

Within Batch	LQC: 2.77 % and HQC: 1.59 %
% Change	LQC: 5.42 % and HQC: 1.20 %

Table No. 26 : Auto Sampler Stability for Ezetimibe (124 Hrs 38 Min)

Quality Control ID	Fresh LQC	Fresh HQC	Stability LQC	Stability HQC
Nominal concentration (ng/mL)	1.205	200.810	1.231	205.127
Acceptance Range	1.024	170.689	1.046	174.358
	1.386	230.932	1.416	235.896
Back Calculated Concentration (ng/mL)	1.199	201.630	1.317	201.103
	1.206	201.062	1.320	207.682
	1.200	200.833	1.240	208.436
	1.270	199.809	1.329	208.468
	1.212	208.691	1.314	209.713
	1.174	208.252	1.266	210.119
Mean	**1.2102**	**203.3795**	**1.2977**	**207.5868**
SD	**0.03205**	**3.99044**	**0.03591**	**3.30088**
% CV	**2.65**	**1.96**	**2.77**	**1.59**
% Change			**5.42**	**1.20**

4. Wet Extract Bench Top Stability

The wet extract bench top stability of analyte for low and high quality control samples were evaluated by analyzing them using a set of freshly spiked and prepared calibration curve standards. The stability samples were kept on bench (at Room temperature).

Based on the results, it was concluded that the analyte is stable at bench top (Wet extract) for 09 Hrs 04 Min.

Acceptance Criteria:

The % change should not be more than ±15% while comparing the mean of back calculated concentration of stability samples against the nominal concentration.

Within Batch Precision	LQC: 2.87 % and HQC: 3.21%
% Change	LQC: 5.56 % and HQC: 7.15 %

Table No. 27 : Wet extract Bench top stability for Ezetimibe (09 Hrs 04 Min)

Quality Control ID	Fresh LQC	Fresh HQC	Stability LQC	Stability HQC
Nominal Concentration (ng/mL)	1.205	200.810	1.231	205.127
Acceptance Range	1.024	170.689	1.046	174.358
	1.386	230.932	1.416	235.896
Back Calculated Concentration (ng/mL)	1.199	201.630	1.307	212.230
	1.206	201.062	1.270	228.274
	1.200	200.833	1.288	219.604
	1.270	199.809	1.251	210.692
	1.212	208.691	1.331	224.972
	1.174	208.252	1.350	222.994
Mean	**1.2102**	**203.3795**	**1.2995**	**219.7943**
SD	**0.03205**	**3.99044**	**0.03729**	**7.05774**
% CV	**2.65**	**1.96**	**2.87**	**3.21**
% Change			**5.56**	**7.15**

5. Freeze thaw stability

To evaluate freeze thaw stability, sufficient individual aliquots of low and high quality control samples were stored in the freezer maintained at -65 ± 10°C. After at least 24 hours of freezing, six different aliquots of low and high quality control samples (stability samples) were retrieved from the freezer and thawed unassisted on bench at room temperature. This stability samples were refrozen for at least 12 hours under the same conditions. The freeze-thaw cycle was repeated for four more times, thereby making the samples undergo five freeze thaw cycles. After completion of fifth freeze thaw cycle, stability samples were processed along with a set of freshly spiked and prepared calibration curve standards and quality control samples (six different aliquots of low and high quality control samples) and analyzed in a single run.

Based on the results, it was concluded that Ezetimibe was stable after five freeze thaw cycle.

Within Batch Precision	LQC: 4.97 % and HQC: 1.42 %
% Change	LQC: -1.12 % and HQC:-0.33 %

Acceptance Criteria:

The % change should not be more than ± 15% while comparing the mean of back calculated concentration of stability samples against the nominal concentration.

Table No. 28 : Freeze Thaw Stability for Ezetimibe after 5th Cycle

Quality Control ID	Fresh LQC	Fresh HQC	Stability LQC	Stability HQC
Nominal Concentration (ng/mL)	1.205	200.810	1.231	205.127
Acceptance Range	1.024	170.689	1.046	174.358
	1.386	230.932	1.416	235.896
Back Calculated Concentration (ng/mL)	1.199	201.630	1.167	206.384
	1.206	201.062	1.193	200.039
	1.200	200.833	1.299	207.247
	1.270	199.809	1.239	207.031
	1.212	208.691	1.264	202.545
	1.174	208.252	1.141	203.456
Mean	**1.2102**	**203.3795**	**1.2172**	**204.4503**
SD	**0.03205**	**3.99044**	**0.06045**	**2.90858**
% CV	**2.65**	**1.96**	**4.97**	**1.42**
% Change			**-1.12**	**-0.33**

6. Bench Top Stability

Bench top stability experiment was designed and conducted to cover the laboratory handling conditions that are expected for study samples.

The bench top stability of Ezetimibe for low and high quality control samples were evaluated by analyzing them using a set of freshly spiked and prepared calibration curve standard.

The Stability samples were kept on bench at room temperature.

Based on the results, it was concluded that the analyte is stable in plasma at room temperature for 10 Hrs 55 Min.

Within Batch Precision	LQC: 1.91 % and HQC: 2.91 %
% Change	LQC: -2.53% and HQC: 0.02 %

Acceptance Criteria:

The % change should not be more than ± 15% while comparing the mean of back calculated concentration of stability samples against the nominal concentration.

Table No. 29 : Bench top stability for Ezetimibe (10Hrs 55 Min)

Quality Control ID	Fresh LQC	Fresh HQC	Stability LQC	Stability HQC
Nominal Concentration (ng/mL)	1.205	200.810	1.231	205.127
Acceptance Range	1.024	170.689	1.046	174.358
	1.386	230.932	1.416	235.896
Back Calculated Concentration ng/mL	1.199	201.630	1.242	201.324
	1.206	201.062	1.190	197.628
	1.200	200.833	1.190	206.700
	1.270	199.809	1.210	210.461
	1.212	208.691	1.182	201.734
	1.174	208.252	1.185	213.173
Mean	**1.2102**	**203.3795**	**1.1998**	**205.1700**
SD	**0.03205**	**3.99044**	**0.02286**	**5.96428**
% CV	**2.65**	**1.96**	**1.91**	**2.91**
% Change			**-2.53**	**0.02**

7. Dry Extract Stability:

Dry Extract Stability was designed and conducted to cover the laboratory handling conditions that are expected for study samples.

The Dry Extract Stability of Ezetimibe for low and high quality control samples were evaluated by analyzing them using a set of freshly spiked and prepared calibration curve standard.

The Stability samples were kept in refrigerator.

Based on the results, it was concluded that the analyte is stable in dry extract at refrigerator temperature (5±3ºC) for 52 Hrs.

Within Batch Precision	LQC: 2.45 % and HQC: 2.46 %
% Change	LQC: 4.26 % and HQC: 1.59 %

Acceptance Criteria:

The % change should not be more than ± 15% while comparing the mean of back calculated concentration of stability samples against the nominal concentration.

Table No.30: Dry Extract Stability for Ezetimibe (52 Hrs)

Quality Control ID	Fresh LQC	Fresh HQC	Stability LQC	Stability HQC
Nominal Concentration (ng/mL)	1.205	200.810	1.231	205.127
Acceptance Range	1.024	170.689	1.046	174.358
	1.386	230.932	1.416	235.896
Back Calculated Concentration ng/mL	1.199	201.630	1.302	209.841
	1.206	201.062	1.288	200.418
	1.200	200.833	1.277	204.227
	1.270	199.809	1.332	212.818
	1.212	208.691	1.245	213.597
	1.174	208.252	1.257	209.487
Mean	**1.2102**	**203.3795**	**1.2835**	**208.3980**
SD	**0.03205**	**3.99044**	**0.03144**	**5.11687**
% CV	**2.65**	**1.96**	**2.45**	**2.46**
% Change			**4.26**	**1.59**

8. Evaporator Stability

Evaporator Stability was designed and conducted to cover the laboratory handling conditions that are expected for study samples.

The Evaporator Stability of Ezetimibe for low and high quality control samples were evaluated by analyzing them using a set of freshly spiked and prepared calibration curve standard.

The Stability samples were kept at 40ºC in Evaporator.

Based on the results, it was concluded that the analyte is stable in evaporator at 40ºC for 02 Hrs 03 Min.

Within Batch Precision	LQC: 4.39 % and HQC: 4.23 %
% Change	LQC: 2.33 % and HQC: -2.10 %

Acceptance Criteria:

The % change should not be more than ± 15% while comparing the mean of back calculated concentration of stability samples against the nominal concentration.

Table No. 31 : Evaporator Stability for Ezetimibe (02 Hrs 03 Min)

Quality Control ID	Fresh LQC	Fresh HQC	Stability LQC	Stability HQC
Nominal Concentration (ng/mL)	1.205	200.810	1.231	205.127
Acceptance Range	1.024	170.689	1.046	174.358
	1.386	230.932	1.416	235.896
Back Calculated Concentration ng/mL	1.199	201.630	1.305	201.012
	1.206	201.062	1.308	208.225
	1.200	200.833	1.258	206.884
	1.270	199.809	1.167	193.585
	1.212	208.691	1.223	207.471
	1.174	208.252	1.290	187.689
Mean	**1.2102**	**203.3795**	**1.2585**	**200.8110**
SD	**0.03205**	**3.99044**	**0.05520**	**8.49290**
% CV	**2.65**	**1.96**	**4.39**	**4.23**
% Change			**2.23**	**-2.10**

9. Long term stability of drug in matrix

To determine long term stability of Ezetimibe in human plasma, the quality control samples were prepared and stored in deep freezers maintained at -65 ± 10°C. Analysis has been carried out after the 85 days of storage. In the below tables data for long term stability for the analyte is provided.

Table No. 32 : Long Term Stability

Quality Control ID	Fresh LQC	Fresh HQC	Stability LQC	Stability HQC
Nominal Concentration (ng/mL)	1.205	200.810	1.231	205.127
Acceptance Range	1.024	170.689	1.046	174.358
	1.386	230.932	1.416	235.896
Back Calculated Concentration (ng/mL)	1.199	201.630	1.167	206.384
	1.206	201.062	1.193	200.039
	1.200	200.833	1.299	207.247
	1.270	199.809	1.239	207.031
	1.212	208.691	1.264	202.545
	1.174	208.252	1.141	203.456
Mean	**1.2102**	**203.3795**	**1.2172**	**204.4503**
SD	**0.03205**	**3.99044**	**0.06045**	**2.90858**
% CV	**2.65**	**1.96**	**4.97**	**1.42**
% Change			**-1.12**	**-0.33**

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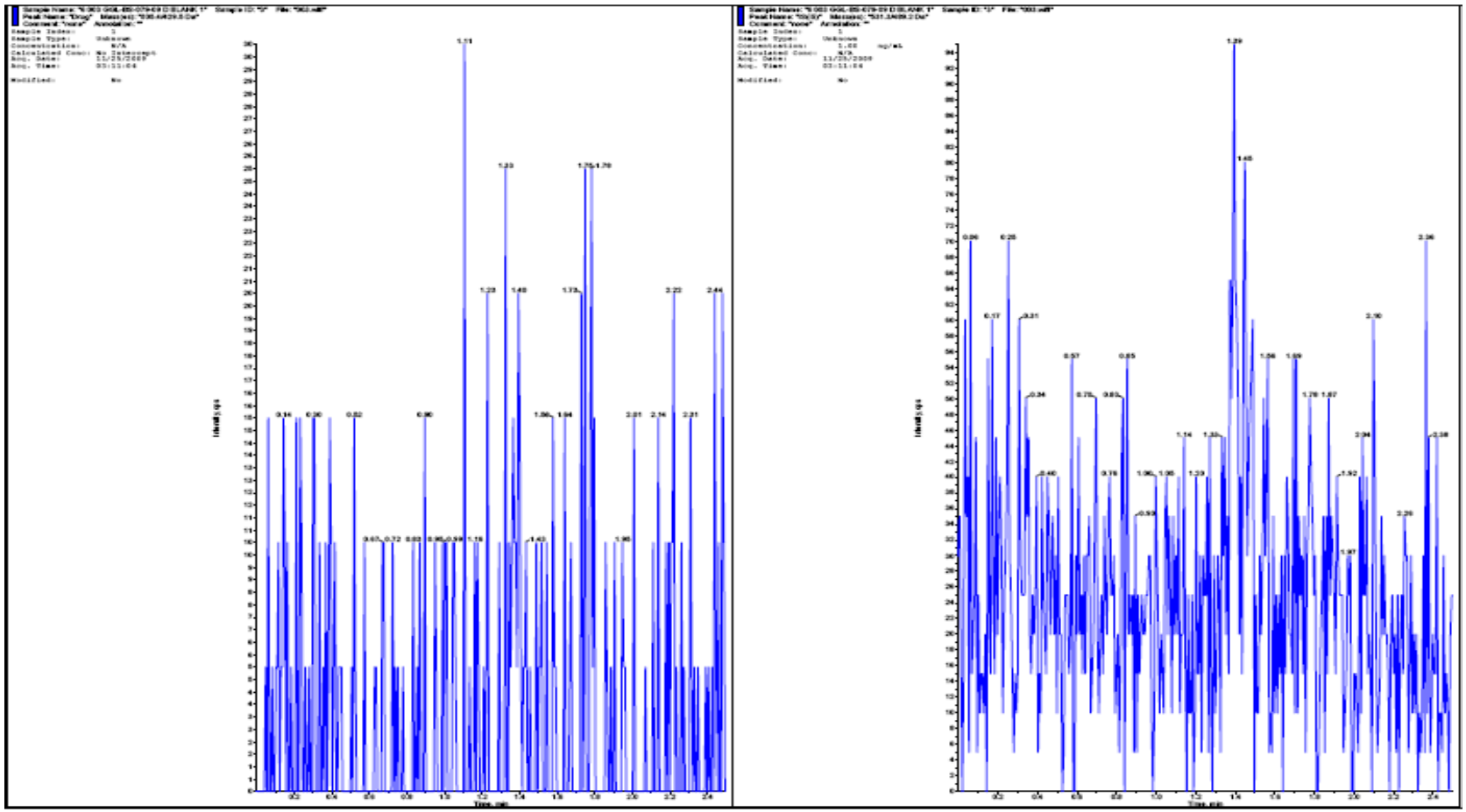

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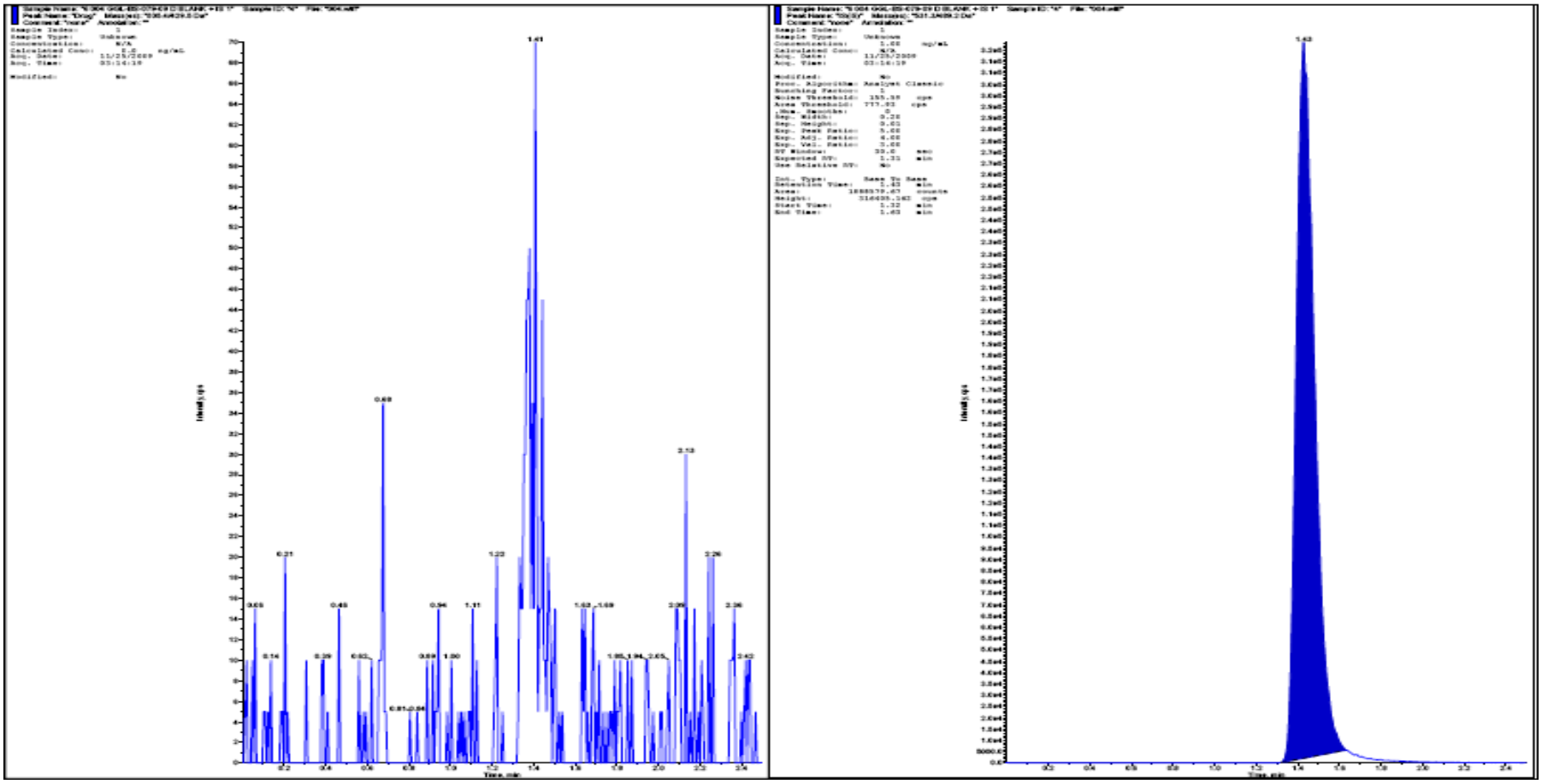

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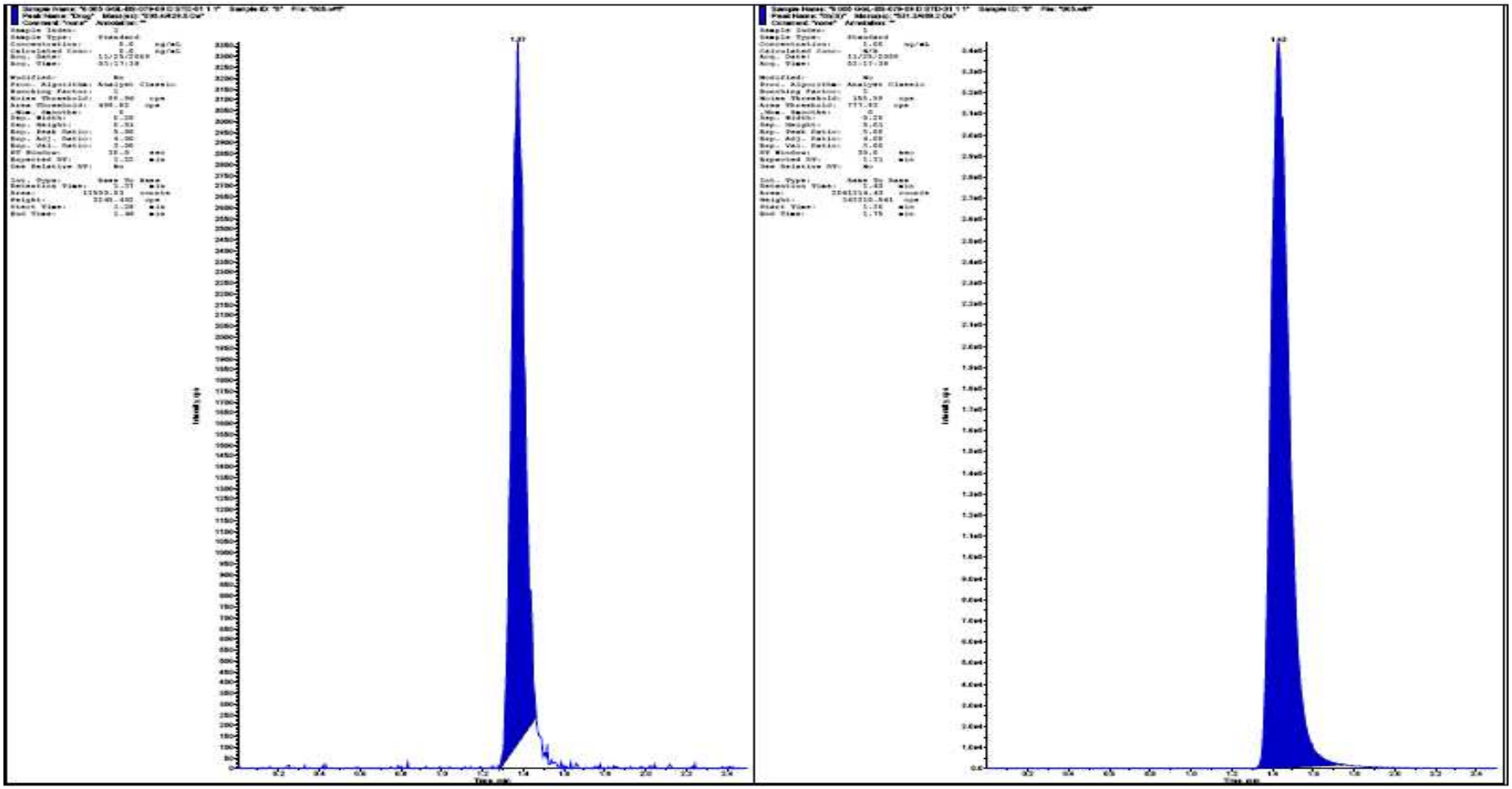

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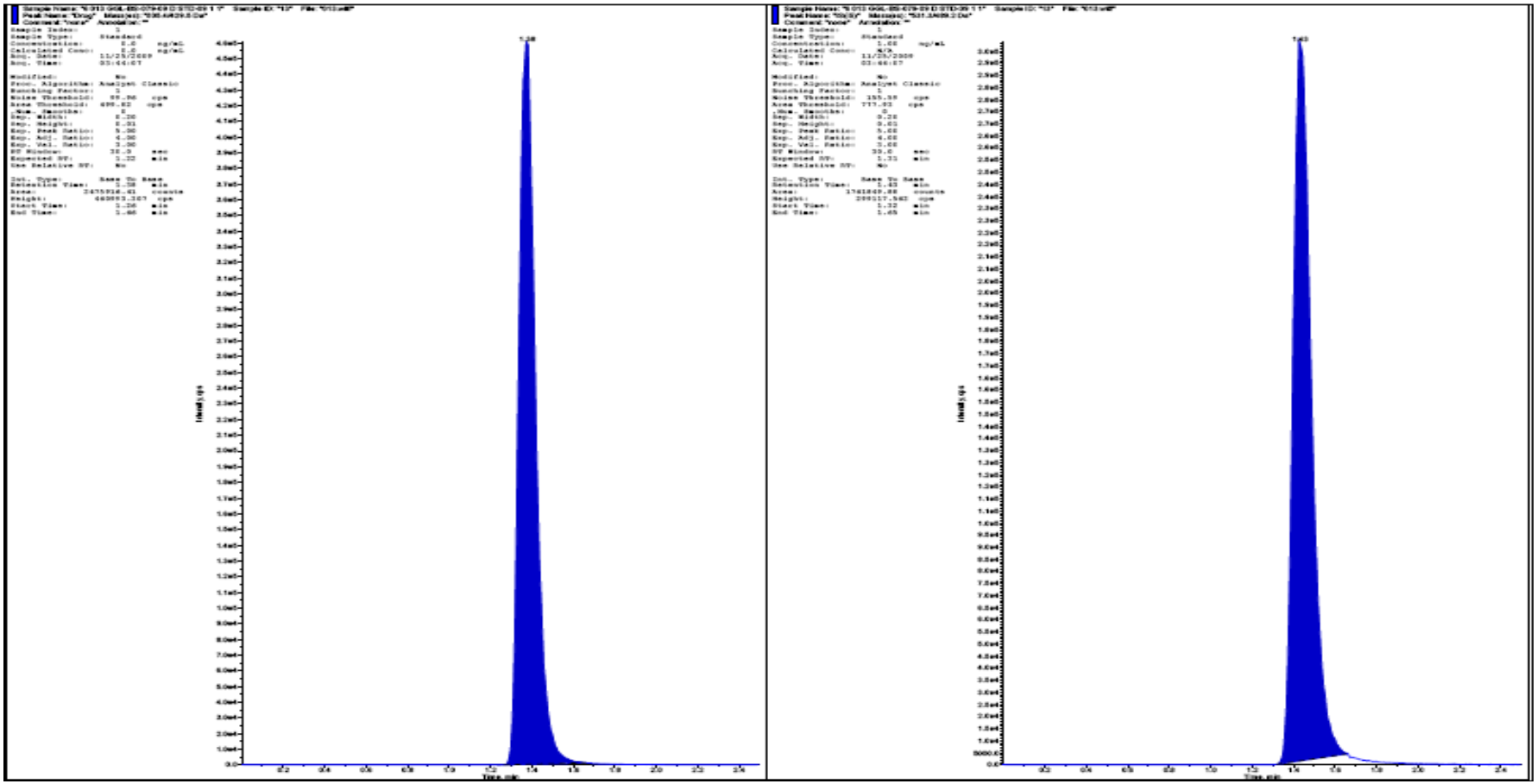

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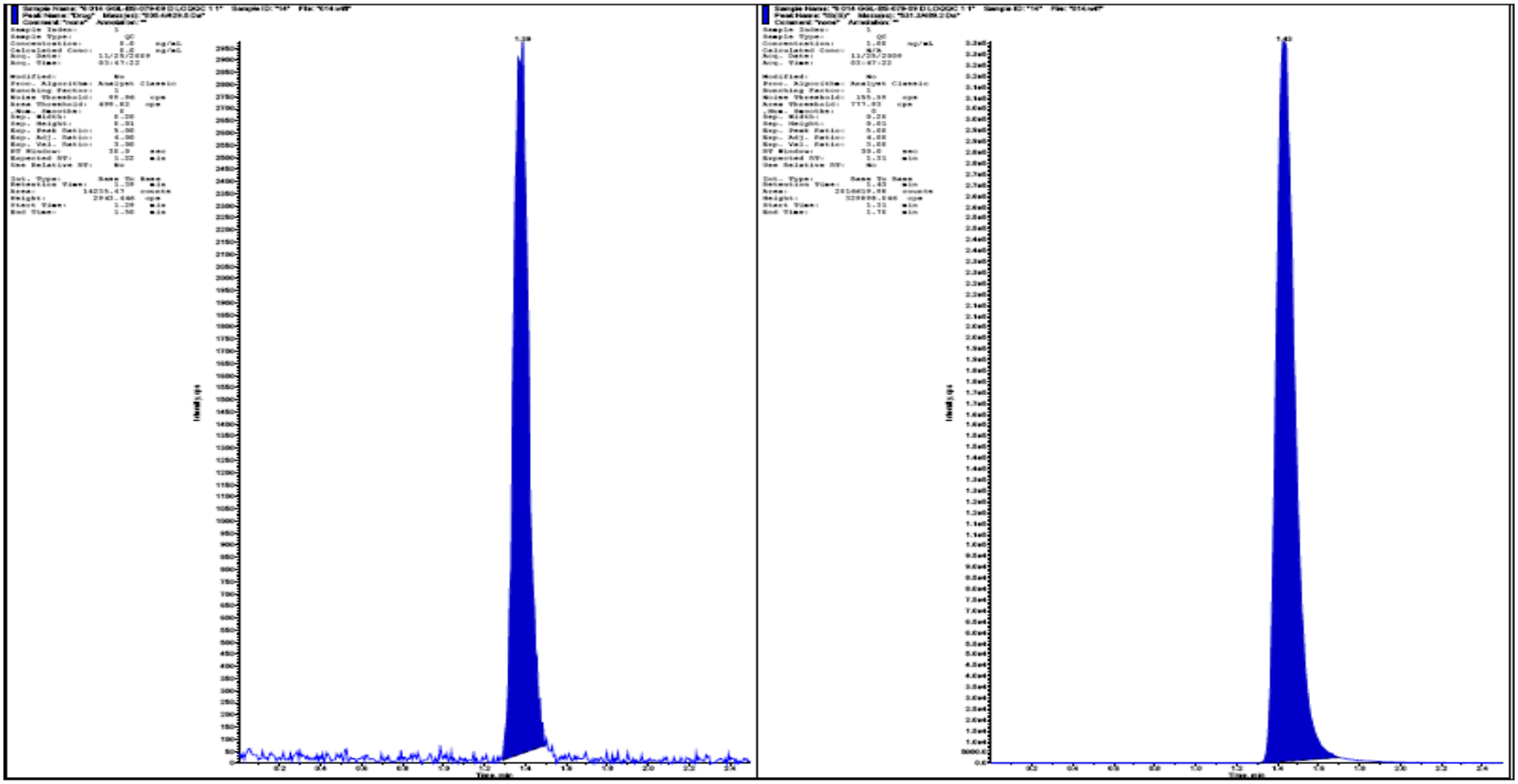

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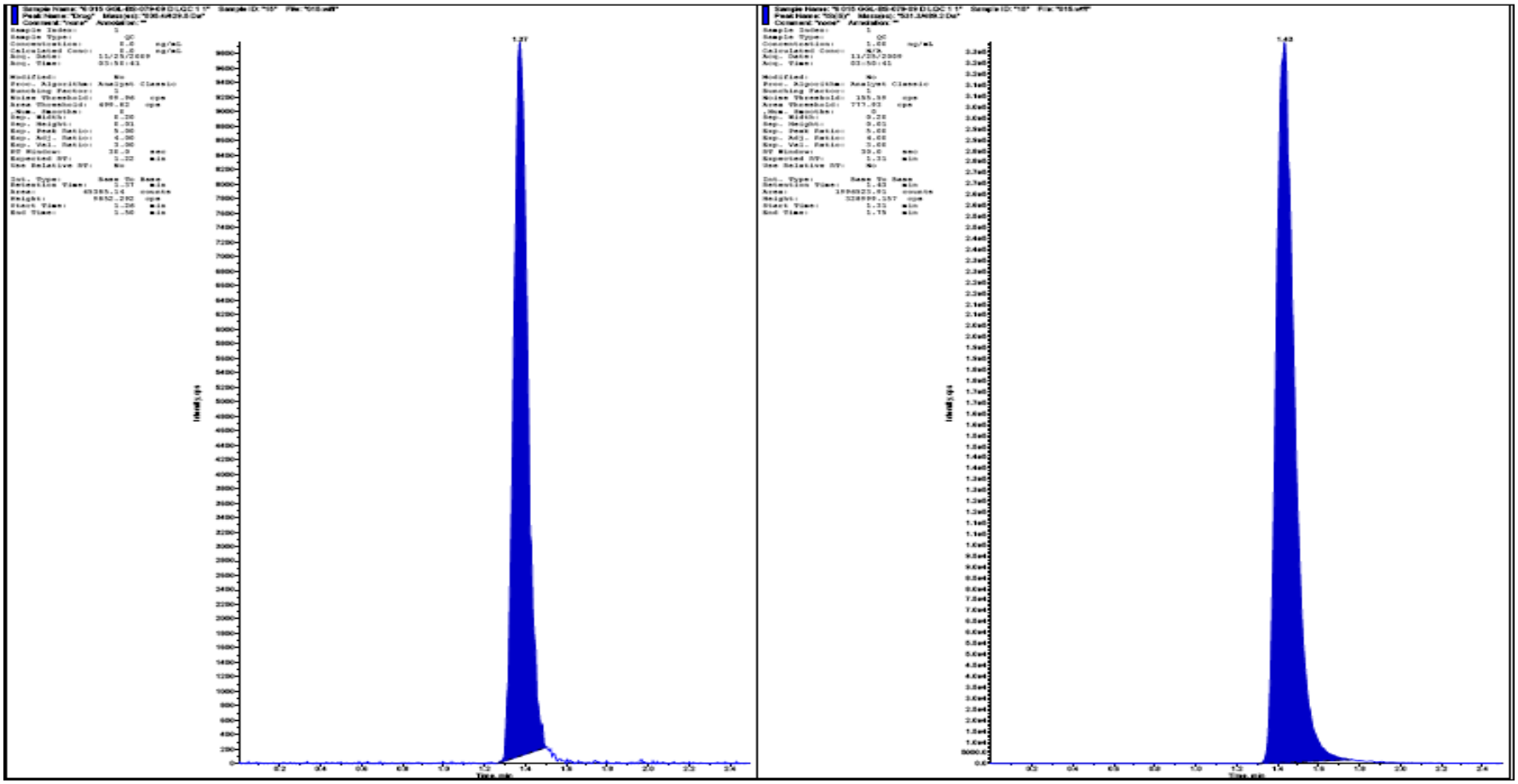

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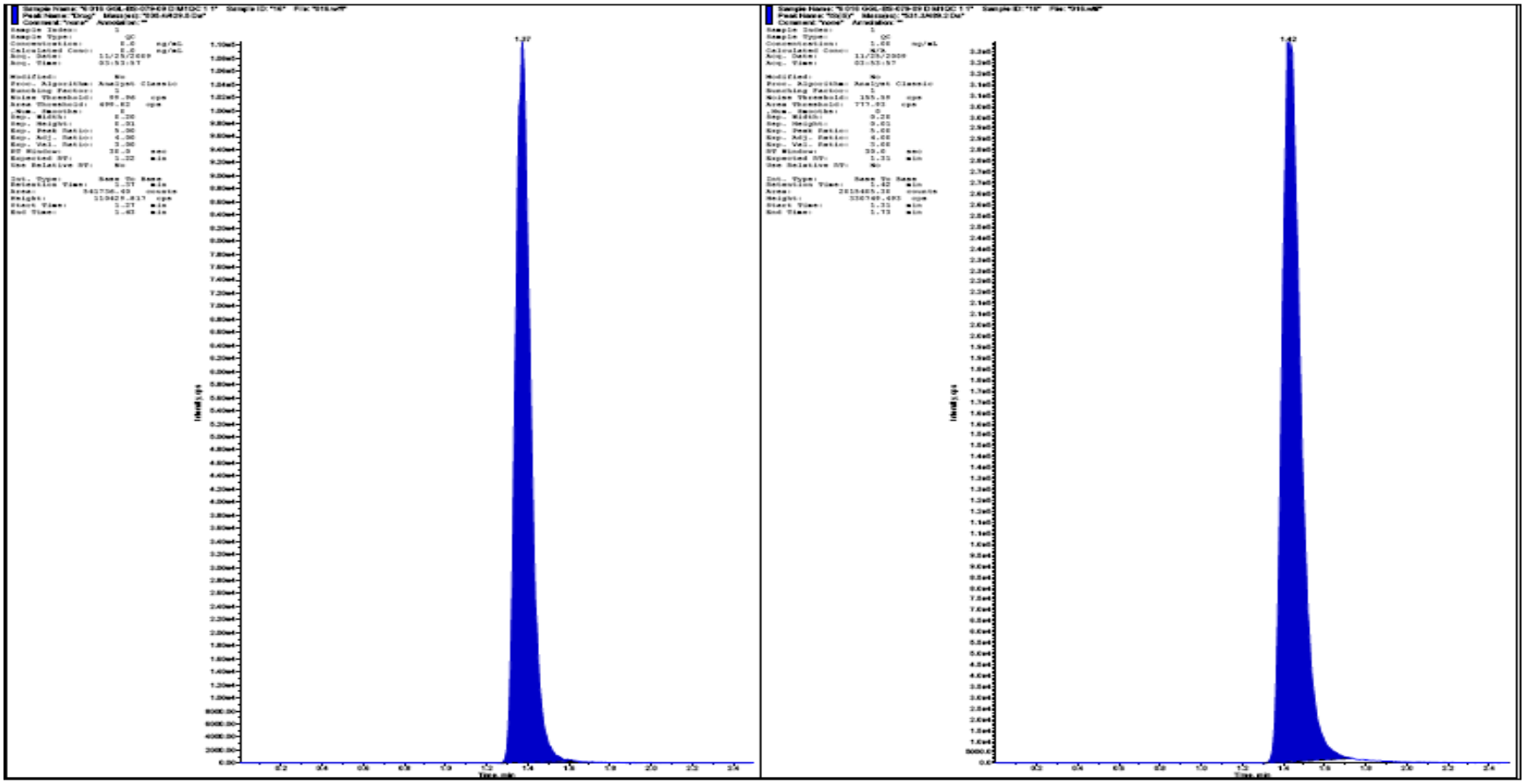
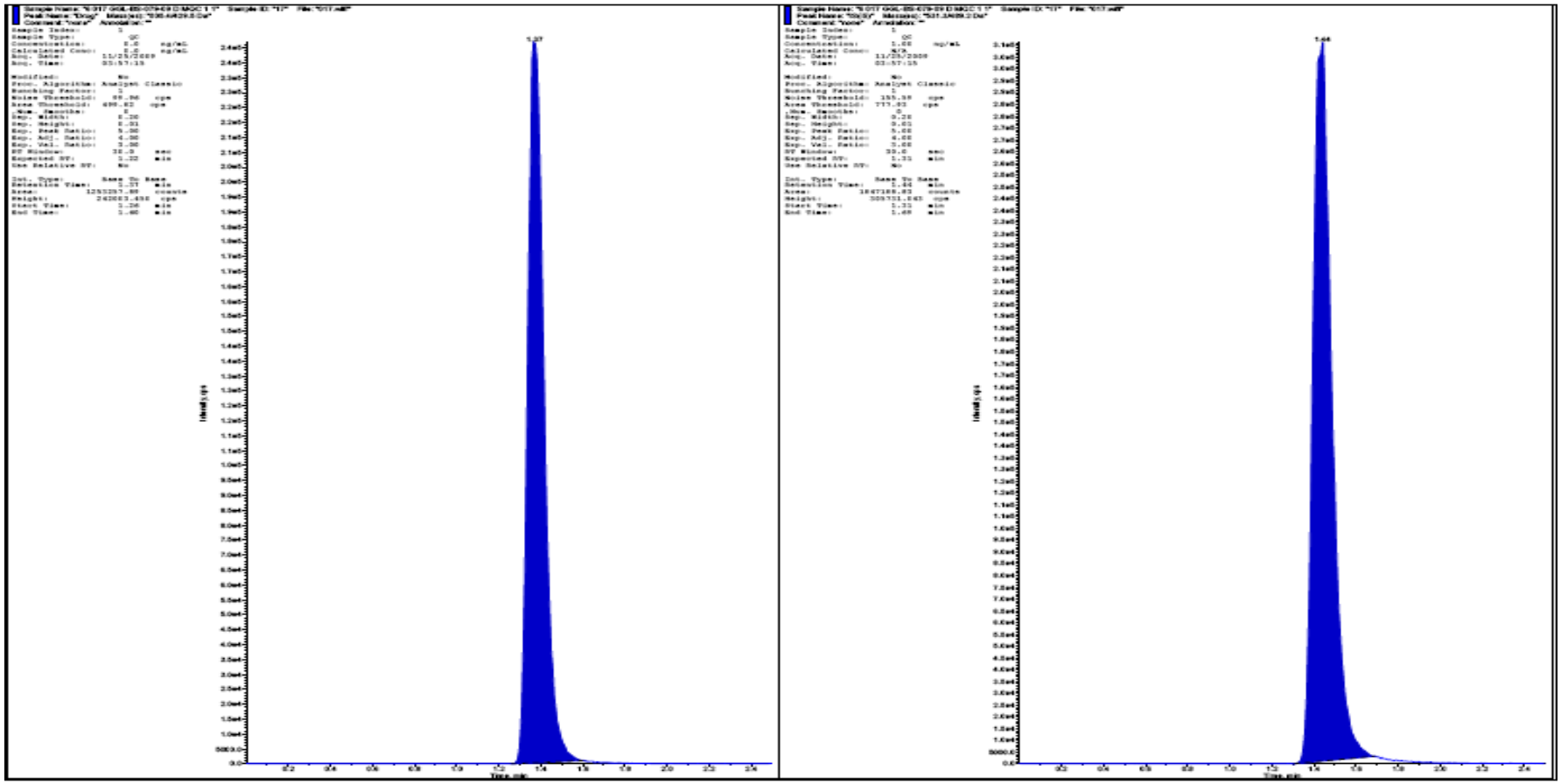
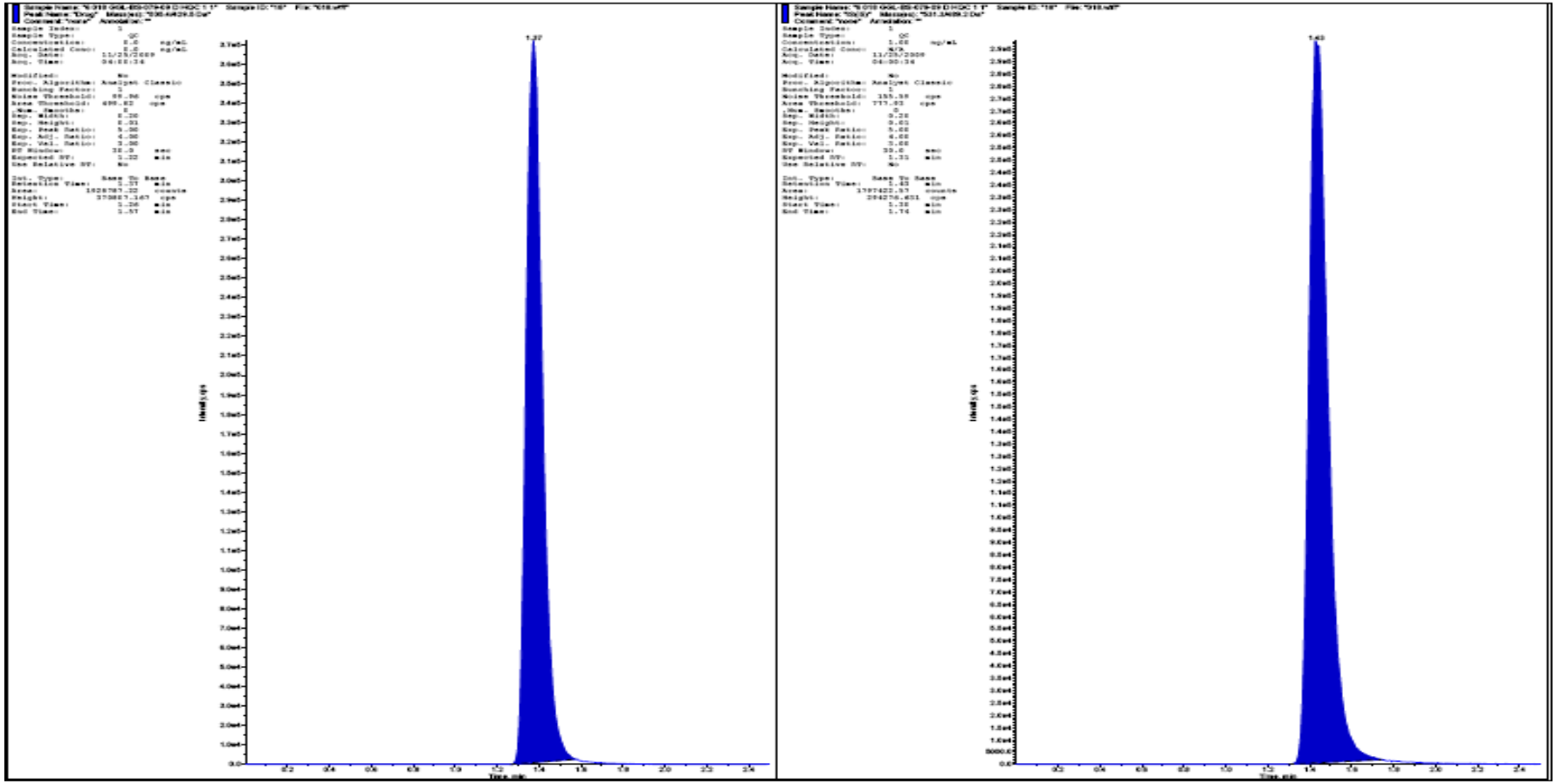


CHROMATOGRAM – 08 CHROMATOGRAM FOR MQC



CHROMATOGRAM – 09 CHROMATOGRAM FOR HQC



SUMMARY AND CONCLUSION

- A simple, specific, rapid and sensitive analytical method for the determination of Ezetimibe in human plasma has been developed.

- Most of the analytical methods reported, for quantization of Ezetimibe individually or simultaneously from human plasma, require laborious extraction procedure like Solid Phase extraction, long run time and high quantification limit.

- The presented method provided excellent specificity and linearity with a limit of quantification of 0.503ng/ml for Ezetimibe. Although it was possible to go down much further in the LLOQ determination of the drug, the results obtained post subject analysis show that the LLOQ values selected are sufficient enough to give data for calculation of the required pharmacokinetic data and establish bioequivalence.

- The other major advantage of this method over all those referenced is the short run time of 1.96 min which allows the quantization of over 200 plasma samples per day.

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GLOSSARY

Accuracy: The degree of closeness of the determined value to the nominal or known true value under prescribed conditions. This is sometimes termed trueness.

Analyte: A specific chemical moiety being measured, this can be intact drug, biomolecule or its derivative, metabolite, and/or degradation product in a biologic matrix.

Analytical run (or batch): A complete set of analytical and study samples with appropriate number of standards and QCs for their validation. Several runs (or batches) may be completed in one day, or one run (or batch) may take several days to complete.

Blank: A sample of a biological matrix to which no analytes have been added that is used to assess the specificity of the bioanalytical method.

Biological matrix: A discrete material of biological origin that can be sampled and processed in a reproducible manner. Examples are blood, serum, plasma, urine, feces, saliva, sputum, and various discrete tissues.

Cross-validation: Comparison validation parameters of two bioanalytical methods.

Calibration standard: A biological matrix to which a known amount of analyte has been added or spiked. Calibration standards are used to construct calibration curves from which the concentrations of analytes in QCs and in unknown study samples are determined.

Full validation: Establishment of all validation parameters to apply to sample analysis for the bioanalytical method for each analyte.

Internal standard: Test compound(s) (e.g. structurally similar analog, stable labeled compound) added to both calibration standards and samples at known and constant concentration to facilitate quantification of the target analyte(s).

Limit of detection (LOD): The lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise.

Lower limit of quantification (LLOQ): The lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

Matrix effect: The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.

Method: A comprehensive description of all procedures used in sample analysis.

Precision: The closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

Partial validation: Modification of validated bioanalytical methods that do not necessarily call for full revalidation.

Processed: The final extract (prior to instrumental analysis) of a sample that has been subjected to various manipulations (e.g., extraction, dilution, concentration).

Quantification range: The range of concentration, including ULOQ and LLOQ that can be reliably and reproducibly quantified with accuracy and precision through the use of a concentration-response relationship.

Quality control sample (QC): A spiked sample used to monitor the performance of a Bioanalytical method and to assess the integrity and validity of the results of the unknown Samples analyzed in an individual batch.

Recovery: The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.

Reproducibility: The precision between two laboratories. It also represents precision of the method under the same operating conditions over a short period of time.

Sample: A generic term encompassing controls, blanks, unknowns, and processed samples, as described below:

Selectivity: The ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present. These could include metabolites, impurities, dégradants, or matrix components.

Stability: The chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

Standard curve: The relationship between the experimental response value and the analytical concentration (also called a calibration curve).

System suitability: Determination of instrument performance (e.g., sensitivity and chromatographic retention) by analysis of a reference standard prior to running the analytical batch.

Upper limit of quantification (ULOQ): The highest amount of an analyte in a sample that can be quantitatively determined with precision and accuracy.